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THE UNIVERSITY OF ALBERTA

# Characterization of Bacteriophage L1 isolated from *Bordetella bronchiseptica* AG1-L.

(C)

by

Anona Lukawiecki

A thesis

submitted to The Faculty of Graduate Studies and Research

in partial fulfilment of the requirements for the degree of

Master of Science

in

Department of Medical Microbiology and Infectious Diseases

Edmonton, Alberta

(Fall 1988)

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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characterization of Bacteriophage L1 isolated from *Bordetella bronchiseptica* submitted by Anona Lukawiecki in partial fulfilment of the requirements for the degree of Master of Science in Bacteriology.

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Date: May 26, 1988

## Abstract

A bacteriophage (L1) was isolated from spontaneous clear plaques in a lawn of virulent (monkey) *Bordetella bronchiseptica*. The bacteriophage L1 was characterized in accordance with the guidelines established by the International Committee for the Taxonomy of Viruses (I.C.T.V.). Morphologically, bacteriophage L1 is a tailed, contractile bacteriophage with an icosahedral head. The buoyant density of bacteriophage L1 in cesium chloride was found to be 1.50 g/ml. The nucleic acid of bacteriophage L1 is a single piece of linear double stranded DNA with no evidence of modified bases. Restriction enzyme analysis revealed the genome to be approximately 50 kb in size. Bacteriophage L1 is sensitive to 1 M sodium chloride solutions and temperatures in excess of 4°C for extended periods of time. Host range experimentation revealed that bacteriophage L1 produces lytic infection in strains of *B. parapertussis* and certain strains of *B. bronchiseptica*, however, *B. pertussis* strains tested showed no lytic infection. In addition to the guidelines for bacteriophage classification issued by the I.C.T.V., protein patterns of bacteriophage L1 compared with bacteriophage T4 and λ were produced by SDS-polyacrylamide gel electrophoresis of [$^{125}$I]-labelled bacteriophage. The resulting protein pattern of bacteriophage L1 was distinct from those of bacteriophage T4 and λ. Autoradiography and staining with Coomassie blue revealed four major bands at $M_r$ = 35K, 33K, 18.5K, and 17K. Furthermore, all visualized protein bands were surface-labelled and therefore presumably exposed on the surface of the bacteriophage. To screen other members of the *Bordetella* species for bacteriophage homologous to bacteriophage L1, colony blot hybridization was conducted on several strains of *B. pertussis*, *B. parapertussis*,

and *B. bronchiseptica* using nick-translated bacteriophage L1 DNA as a probe. Hybridization was most prominant within the strains of *B. bronchiseptica* therefore, further screening was done in the form of dot blot hybridization and finally Southern blot hybridization on certain strains of *B. bronchiseptica*. Three strains of *B. bronchiseptica* isolated from the same bordetellosis outbreak as strain AG1-L carried homologous bacteriophage. The bacteriophage detected by Southern blot hybridization appeared to be in the non-lysogenic or non-integrated form. In addition, one other strain of *B. bronchiseptica* produced a band in the Southern blot that weakly hybridized with bacteriophage L1 but did not correspond to any bands in the purified bacteriophage L1 DNA. This strain may possibly carry a lysogenic bacteriophage which is homologous with bacteriophage L1. The possible relevence of bacteriophage L1 is also discussed.

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## List of Abbreviations

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| *B. bronchiseptica* | *Bordetella bronchiseptica* |
| *B. parapertussis* | *Bordetella parapertussis* |
| *B. pertussis* | *Bordetella pertussis* |
| BGA | Bordet-Gengou agar |
| BSA | bovine serum albumin |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| CFU/ml | colony forming units per milliliter |
| Ci/mM | Curies per milliMole |
| CPM | counts per minute |
| CSM | cyclodextrin solid medium |
| ° C | degrees Celsius |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| g | gram |
| g/ml | gram per milliliter |
| x g | times gravity (centrifugal force) |
| G protein | guanine-nucleotide-binding proteins |
| $G_i$ | inhibitory guanine-nucleotide-binding protein |
| GTP | guanosine triphosphate |
| I.C.T.V. | International Commitee for the Taxonomy of Viruses |
| hr. | hour |

| | |
|---|---|
| IgE | immunoglobulin E |
| K antigen | capsular antigen |
| kb | kilo base |
| kD | kilo Dalton |
| λ | bacteriophage Lambda |
| L-sloppy agar | Lauria-Bertani sloppy agar |
| LA | Lauria-Bertani agar |
| LB | Lauria-Bertani medium |
| $LD_{50}$ | lethal dose for 50% of the test population |
| LPS | lipopolysaccharide |
| M | Molar |
| min. | minute |
| µg | microgram |
| µl | microliter |
| µm | micrometer |
| mM | milliMolar |
| mg | milligram |
| ml | milliliter |
| mm | millimeter |
| N | Normal |
| nm | nanometer |
| O antigen | somatic antigen |
| OD | optical density |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |

| | |
|---|---|
| PEGppt | polyethylene glycol precipitate |
| PFU/ml | plaque forming units per milliliter |
| psi | pounds per square inch |
| RNA | ribonucleic acid |
| rpm | rotations per minute |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SSA | Stainer-Scholte agar |
| SSB | Stainer-Scholte broth |
| SSPE | SSPE buffer |
| SSC | saline sodium citrate buffer |
| t | time |
| T4 | bacteriophage T4 |
| TA | Tris-Acetate |
| TB | Tris-Borate |
| TE | Tris-EDTA |
| TCA | trichloroacetic acid |
| ™ | trade mark |
| $T_M$ | temperature at which 50% of DNA in a sample dissociates |
| TSA | trypticase soy agar |
| TSB | trypticase soy broth |
| uv | ultraviolet |
| v/v | volume per volume |

| | |
|---|---|
| wt./vol. | weight per volume |

# 1.0 Introduction

**1.1 The Genus *Bordetella*.** The microorganisms placed within the genus *Bordetella* are bacterial, respiratory pathogens. Biologically, the organisms are small, strictly aerobic, Gram negative coccobacilli that localize and proliferate on the respiratory epithelial cells of mammalian or avian hosts (Goodnow 1980, Musser *et al.* 1986). In addition to these similar biological characteristics, all members of the genus *Bordetellae* are serologically related (see Table 1) in that all possess cross reactive K and O antigens ( Pittman 1984, Eldering *et al.* 1957).

**1.1.1 The *Bordetella* species.** The genus *Bordetella* is subdivided into four species on the basis of phenotypic characteristics (see Table 2) including growth on Bordet-Gengou agar and peptone agar, motility, and biochemical capacities such as citrate utilization, nitrate reduction, and urease and oxidase activity (Pittman 1984, Goodnow 1980 ). *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* are further distinguished serologically (see Table 1), by virtue of species specific antigens also termed agglutinogens (Eldering *et al.* 1957). Eldering *et al.* (1957) described the fourteen heat-labile "K" antigens or agglutinogens by their ability to produce antisera in rabbits that caused the agglutination of specific *Bordetella* cells. Eldering *et al.* found that three agglutinogens, factors 1, 12, and 14, were species specific for *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* respectively, and could be used to differentiate between the three species. More recent studies have shown that agglutinogens 2 and 3 are associated with fimbriae rather than "K" antigens (see section 1.2.5). The studies of Eldering *et al.* also predate the recognition of the fourth species, *B. avium* within the genus *Bordetella*. Available serological data for *B. avium* are incomplete. However,

**Table 1**: Differential and common antigens of phase I strains of the species of the genus *Bordetella*.

| Antigens | *B. pertussis* | *B. parapertussis* | *B. bronchiseptica* |
|---|---|---|---|
| K-Antigens | | | |
| Common to genus: | | | |
| Factor 7 | + | + | + |
| Species Specific: | | | |
| Factor 1 | + | - | - |
| Factor 14 | - | + | - |
| Factor 12 | - | - | + |
| Other factors occurring in one or more strains: | | | |
| Factors 2,3,4,5,6,13 | + | - | - |
| Factors 8,9,10 | - | + | - |
| Factors 8,9,10,11,13 | - | - | + |
| O-Antigen, common | + | + | + |

(Adapted from Pittman 1984)

**Table 2**: Differentiation between *Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica*, and *Bordetella avium.*

| Characteristics | *B. pertussis* | *B. parapertussis* | *B. bronchiseptica* | *B. avium* |
|---|---|---|---|---|
| Motility | - | - | - | - |
| Growth on BGA: | | | | |
| 1-2 days | - | + | + | + |
| 3-6 days | + | + | + | + |
| Growth on peptoneagar: | | | | |
| Phase I | - | + | + | + |
| Phase IV | + | + | + | + |
| Browning | - | + | - | + |
| Growth on MacConkey agar: | - | + | + | + |
| Citrate Utilization: | - | + | + | + |
| Nitrate Utilization: | - | - | + | + |
| Urease | - | + | + | - |
| Oxidase | + | - | + | + |

( Adapted from Pittman 1984)

Hinz *et al.* (1979) reported *B. avium* isolates had an O antigen which cross reacted with an O antigen of a strain of *B. bronchiseptica* isolated from a pig.

**1.1.2 The Mammalian Pathogens.** With the exception of *B. avium* , which was isolated from domestic fowl with respiratory infection (Kersters *et al.* 1984 , Hinz *et al.* 1979 ), all species of *Bordetella* are mammalian pathogens. These *Bordetella* species demonstrate preferential adherence to mammalian ciliated epithelial cells. *B. parapertussis* and *B. pertussis* preferentially adhere to human cells however, *B. bronchiseptica* strains preferentially adhere to nonhuman mammalian ciliated cells of rabbits, mice, and hamsters (Tuomanen *et al.* 1983). Although man can be infected by all three mammalian pathogens, man is the only known reservoir for just two of the three mammalian pathogens, *B . pertussis* and *B . parapertussis* ( Pittman 1984). This is interesting in the light of reports of carriage of *B. pertussis* in man being low or nil (Linnnemann *et al.* 1968 ).

**1.1.2.1 *Bordetella pertussis*.** In man, the most serious infection is that caused by *B. pertussis*. This organism is the etiological agent of the childhood disease whooping cough or pertussis, which prior to the extensive vaccination program, caused the deaths of many children (Stewart 1984). The disease is characterized by a localized bacterial infection of the upper respiratory tract, specifically the nasopharynx, and the production of toxins which cause the characteristic paroxysmal cough and other systemic sequelae including neurological symptoms, lymphocytosis, hyperinsulinemia, and hypoglycemia.

**1.1.2.2 *Bordetella parapertussis*.** Infection with *B. parapertussis* can lead to subclinical infection or may cause a milder form of pertussis (Linnemann and Perry 1977). In rare cases, infant mortality as a result of pneumonia and encephalapathy caused by *B. parapertussis* infection has been reported (Linnemann and Perry 1977).

Infection with *B. pertussis* and *B. parapertussis* are considered to be immunologically distinct with no induction of cross immunity (Granstrom and Askelof 1982, Lautrop 1958).

**1.1.2.3 *Bordetella bronchiseptica*.** On occasion, man is infected with *B. bronchiseptica* producing a mild respiratory infection, but this is rare and can be traced to persons in contact with infected animals and is therefore considered a zoonotic infection ( Goodnow 1980, Ghosh and Tranter 1979). The major problem of *B. bronchiseptica* infection is in the swine and kennel industry and in laboratory settings, where large numbers of animals are raised in confined areas ( Musser *et al.* 1987, Goodnow 1980 ). The organism is a major etiological agent in swine infectious atrophic rhinnitis and pneumonia ( Bemis and Wilson 1985, Straw *et al.* 1983, Ross *et al.* 1963, Switzer 1956), canine tracheobronchitis or kennel cough (Tischler and Hill 1977, Wright *et al.* 1973), and respiratory infection in such laboratory animals as monkeys, rats, rabbits, ferrets, marmosets, and guinea pigs ( Goodnow 1980, Ferry 1913). The organism is also responsible for epidemic infections in wild animals such as foxes, opossums, racoons, and skunks (Bemis *et al.* 1977, Pittman 1984 ).

**1.1.3 Relatedness of the Mammalian Pathogens.** The three mammalian pathogens, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, are genetically highly related. Kloos *et al.* (1978) reported DNA homologies as determined by DNA/DNA reassociation reactions of 72 to 93% between strains of *B. bronchiseptica* and *B. pertussis* and 88 to 96% between strains of *B. parapertussis* and *B. pertussis*. In addition, Kloos *et al.* (1978) reported the transformation of leucine and tryptophan auxotrophs of *B. pertussis* by DNA of *B. parapertussis* and *B. bronchiseptica* at close to homologous values. Confirmation of the transformation system in *B. pertussis* was done by treatment of *B. pertussis* with N-methyl-N'-nitro-N-nitrosoguanidine to create

auxotrophic mutants which were used as recipients in transformation experiments. Johnson and Sneath (1973) reported in a numerical taxonomic survey that the strains of the genus studied had a cophenetic correlation of 0.96. Musser *et al.* (1986) measured the genetic diversity in natural *Bordetella* populations by analyzing the allelic variations of fifteen enzymes by electrophoretic mobility. Musser found the population structure of *Bordetella* species to be clonal and suggested that the division of the genus *Bordetella* into three mammalian species is not justified when compared to the diversity found within other genera of pathogenic bacteria. Unique to *B. pertussis* is the production of pertussis toxin, a major virulence factor thought responsible for much of the pathogenesis of whooping cough ( Pittman 1984). However, Marchitto *et al.* (1986) reported that the pertussis toxin genes were present in both *B. parapertussis* and *B. bronchiseptica* , but were not expressed in these species. Nicosia and Rappuoli (1987) determined the pertussis toxin genes were arranged in an operon and that weaker promoter sequences in *B. parapertussis* and *B. bronchiseptica* did not allow for the expression of these operons. This suggested a very recent divergence from a common ancestor ( Arico and Rappuoli 1987).

**1.2 Virulence-Associated Factors.** The mammalian pathogens have several properties in common, one of which is, production of similar virulence-associated factors which endow the *Bordetella* species with the ability to attach to and proliferate on the cilia of the naso-epithelium (Tuomanen *et al.* 1983, Bemis and Kennedy 1981, Yokomizo and Shimizu 1979, Muse *et al.* 1978), and then to maintain infection of the host. The second is to undergo phenotypic variation which affects the expression of a number of these shared virulence-associated factors, as shown in *in vitro* experiments of phase variation and antigenic modulation (Peppler and Schrumpf 1984, Weiss and

Falkow 1984, Lacey 1953). There are several virulence-associated factors produced by the *Bordetella* species.

**1.2.1 Dermonecrotic toxin.** Dermonecrotic toxin, produced by all three species and also known as heat-labile toxin, is toxic to mice and guinea pigs and causes dermonecrotic lesions when injected subcutaneously into rabbits (Munoz and Bergman 1977, Nakai *et al.* 1985). The native toxin, purified from *B. bronchiseptica* cultures, shows a mobility of approximately 190,000 kD during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and trypsinized preparations can be reversibly dissociated into two polypeptides (fragment 1 = 75 000 kD and fragment 2 = 118 000 kD) by treatment with urea and dithiothreitol (Kume and Nakai 1987 ). Nakase and Endoh (1984) report the molecular weight of the dermonecrotic toxin of both *B. bronchiseptica* and *B. pertussis* to be 102 000 kD by gel filtration. Antitoxin produced against formalinized *B. bronchiseptica* dermonecrotic toxin has been shown to neutralize the toxins of all three *Bordetella* species (Evans 1940) indicating a similarity in the toxins of the three species. Dermonecrotic toxin activity has been shown to have a positive correlation with the ability of strains of *B. bronchiseptica* to cause respiratory disease in swine (Roop *et al.* 1987, Collings and Rutter 1985, Elias *et al.* 1982) and dogs (Goodnow *et al.* 1983). The role of dermonecrotic toxin in human disease has not been determined (Robinson *et al.* 1985, Weiss *et al.* 1984). Although a potent toxin, the mechanism of toxic action is not known. It has been shown that the addition of dermonecrotic toxin to a mixture of ATP and $Na^+$- $K^+$ ATPase significantly inhibits the conversion of ATP to ADP, a process necessary to prevent vascular muscle contraction (Nakase and Endoh 1984). The contraction of the vascular smooth muscle induces vasoconstriction which has been proposed to cause the dermonecrotic lesions.

**1.2.2 Filamentous haemagglutinin.** Filamentous haemagglutinin can be isolated from the culture supernatant fluids of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (Granstrom and Askelof 1982, Munoz *et al.* 1981, Irons and MacLennan 1979, Morse and Morse 1970). Filamentous haemagglutinin of *B. pertussis* is a heterogeneous population of polypeptides appearing in negatively stained electronmicrographs as fine filaments 2 nm in diameter and 40 -100 nm in length with molecular weight of approximately 200,000 (Irons *et al.* 1983, Arai and Sato 1976). Although it has been implicated in the adherence of *B. pertussis* to mammalian cell surfaces (Gorringe *et al.* 1985, Tuomanen and Weiss 1985), the role of filamentous haemagglutinin in pathogenesis of whooping cough is uncertain. Weiss *et al.* (1984) found that in the infant-mouse model, mutants deficient in production of filamentous haemagglutinin were almost as virulent as the wild-type organisms. The function of filamentous haemaggutinin in other *Bordetella* infections is unknown. Piliation or other attachment factors are important in the pathogenesis of *B. bronchiseptica* infections (Bemis *et al.* 1977). A role for filamentous haemmagglutinin in the pathogenesis of *B. bronchiseptica* is within the realm of reason. Bemis and Plotkin (1982) reported no differences in haemagglutinating capacity of *B. bronchiseptica* strains from different animal isolates. The role of haemagglutinins as accessory attachment factors seems more likely when the host specifity of *B. bronchiseptica* isolates is considered.

**1.2.3 Adenylate cyclase.** Extracellular adenylate cyclase is produced by virulent cultures of the three *Bordetella* species (Endoh *et al.* 1980). Novotny *et al.* (1985) found that adenylate cyclase activity from the culture supernatants of all three *Bordetella* species could be absorbed to the same extent by the same monoclonal immunosorbant. The adenylate cyclase of *B. pertussis* has a molecular weight of 69 000 to 70 600 (Hewlett and Wolff 1976, Hewlett *et al.* 1979) and the enzyme activity is found

largely in association with the cell membrane of the organism in contrast to the largely extracellular location of adenylate cyclase of *B. parapertussis* and *B. bronchiseptica* (Confer and Eaton 1984, Endoh *et al.* 1980, Hewlett *et al.* 1979). However, adenylate cyclase activity was reported in a 68 000 kD protein isolated from the outer membrane of *B. bronchiseptica* (Novotny *et al.* 1985). Novotny and co-workers (1985) could not determine whether the 68 000 kD protein was a component of adenylate cyclase or whether it was an unrelated protein associated with the enzyme. Adenylate cyclase has been proposed as a potent toxin of mammalian cells, in particular phagocytes, by becoming internalized by the target cells and causing the unregulated production of cyclic AMP and the impairment of cellular function (Confer and Eaton 1982, Bourne *et al.* 1971). It has been implicated as a contributing factor in the impairment of host defences seen in *Bordetella* infections as evidenced in reports of the severe impairment of alveolar macrophage activity in rabbits infected with *B. bronchiseptica* and the reported lack of neutrophil response noted in the human whooping cough syndrome (Hoidal *et al.* 1978, Olsen 1975). Mutant strains of *B. pertussis*, deficient in adenylate cyclase activity and haemolysin, could not produce infection in the infant-mouse model (Weiss *et al.* 1984) suggesting the toxin adenylate cyclase may be required in human infection. Mutants deficient solely in adenylate cyclase activity have not yet isolated.

**1.2.4 Haemolysin.** Haemolysin causes the lysis of erythrocytes of many species of animals and has been used along with colonial morphology, as a standard marker of virulence of *Bordetella* species when grown on Bordet-Gengou agar (Peppler and Schrumpf 1984, Peppler 1982). Investigation of the role of haemolysin in *Bordetella* infection has produced rather inconclusive results. Experimental infection of piglets with *B. bronchiseptica* strains expressing the haemolytic activity and colonial

morphology of virulent organisms were not always found to produce infection and disease (Roop *et al.* 1987). The mutant strain of *B. pertussis* deficient in both haemolysin but as well, adenylate cyclase could not produce infection in the infant mouse model (Weiss *et al.* 1984). It is unclear whether both adenylate cyclase and hemolysin are essential to produce infection or only one of the two may be required.

**1.2.5 Agglutinogens.** The agglutinogens are associated with adhesive properties of the *Bordetella* species and in this capacity, deemed as virulence-associated factors (Robinson *et al.* 1987). Eldering *et al.* (1957) described fourteen serologically distinct surface antigens that stimulate the production of antibodies and cause the agglutination of *Bordetella* cells and have been used to serotype the genus *Bordetella*. More recent studies have characterized the agglutinogens as membrane proteins with molecular weights ranging from 10 000 to 23 000 and containing some carbohydrate (Zakharova 1979, Novotny *et al.*. 1979). Agglutinogen 2 and 3 of *B. pertussis* have been demonstrated in association with fimbriae (Ashworth *et al.* 1982, Robinson *et al.* 1987).

**1.2.6 Pertussis Toxin.** Pertussis toxin, also known as lymphocytosis promoting factor, pertussigen, histamine sensitizing factor, or islets activating protein, is the major virulence factor associated with whooping cough and is produced solely by *B. pertussis* (Pittman 1984, Robinson *et al.* 1985). The toxin, a protein of 105 kD, is comprised of five different structural subunits that can be divided into two functional moieties. The A moiety, composed of one subunit, S1, posesses ADP-ribosyltransferase activity. The B moiety, composed of four subunits arranged in two dimers ( {S2 and S4} and {S3 and S4} ) held together with S5, is able to bind to receptors on the eukaryotic cell surface (Sekura *et al.* 1983 , Tamura *et al.* 1982). The genetic information coding for the toxin subunits is arranged in a single operon

(Nicosia *et. al.* 1986, Locht and Keith 1986). The mechanism by which the toxin exerts its effect on eukaryotic cells involves binding to the cell surface with the B moiety and introducing the ADP-ribosyltransferase of the A moiety into the cell. The enzyme ADP-ribosylates the alpha subunits of certain GTP-binding proteins, particularily the $G_i$ regulatory protein involved in the receptor mediated down-regulation of adenylate cyclase. The G proteins are involved in the transduction of signals across the cell membranes through the secondary messenger cAMP (Hsia *et al.* 1984, Katada and Ui 1982). Pertussis toxin has many biological effects such as causing lymphocytosis, histamine sensitization in mice, hyperinsulinemia, hypoglycemia, adjuvanticity (especially for IgE), increased susceptibility to anaphylactic shock, and mitogenicity for lymphocytes (Robinson *et al.* 1985). It causes the release of fatty acids from fat cells, interfers with chemotactic migration, and changes peripheral vascular permeability (Hewlett *et al.* 1983, Munoz *et al.* 1981)

**1.3 Expression of Virulence-Associated Factors.** The ability of the *Bordetella* species to cause disease is conferred through the expression of the genus- and species-specific virulence determinants. All three species, however, have the propensity to undergo phase variation,which among other things includes alteration in the virulence capacities of the organism. This can occur especially on serial passage on laboratory culture media.

**1.3.1 Phase Varation.** Phase variation within the *Bordetella* species, which has been correlated with alterations in colonial morphology, envelope proteins, cytochromes of the respiratory electron transport chains, growth requirements and antibiotic sensitivities, also involves the switching between virulent phase, in which the virulence factors are expressed, and an avirulent phase. The usual direction of the switch is from virulent to avirulent and can be reversible (Leslie and Gardner 1931,

Lawson 1939, Florsdorf *et al.* 1941, Standfast 1951, Kasuga *et al.* 1953, 1954, Ezzel *et al.* 1981, Ezzel *et al.* 1981, Peppler 1982, Peppler and Schrumpf 1984). Leslie and Gardner (1931) described four antigenically distinct stages in phase variation in *B. pertussis* strains: phase I corresponded to toxic organisms as isolated, phase II were passaged organisms but still toxic to guinea pigs, and phase III and phase IV being relatively non-toxic. In the scheme of Leslie and Gardner (1931), the phase variation consisted of an ordered loss of virulence determinants with the end result of a non-toxic phase IV organism. On further investigation, Lawson (1939) suggested that there were several barely distinguishable intermediate forms (phases) and that the process involved a gradual loss of virulence. Parker (1976, 1979) proposed that the phase variation was the result of a random process caused by the accumulation of point mutations and the selection of avirulent mutants due to their enhanced ability to grow in *in vitro* conditions. Avirulent phase variants have been shown to have an increased resistance to fatty acids and certain antibiotics (Dobrogosz *et al.* 1979, Field and Parker 1979, Peppler and Schrumpf 1984). Weiss and Falkow (1984) proposed that virulence determinants can be lost or regained in a single step event. Alternatively, Goldman *et al.* (1987) found that the chromosomal DNA of virulent strains of *B. pertussis* was protected against enzymatic digestion by a modification and that the variation in *B. pertussis* may be caused by changes in the modification of the DNA rather than by mutation.

Nakase (1957) described strains of *B. bronchiseptica* freshly isolated from guinea pigs as phase I organisms that upon subsequent subculture switched to phase II and III. Ocassionally, there was a further phenotypic change that would correspond to phase IV. Peppler and Schrumpf (1984) also found phase change in *B. bronchiseptica* strains.

Kasuga *et al.* (1958) reported phase variation within *B. parapertussis* strains. Three antigenically distinct phases corresponding to phase I, II, and III characteristics of Leslie and Gardner (1931) were observed with phase I organisms being in the pathogenic phase.

**1.3.2 Antigenic Modulation.** In contrast to the heritable, stable changes of phase variation, *B. pertussis, B. parapertussis* , and *B. bronchiseptica* have been shown to undergo reversible alterations in serological composition when cultured on a modified Bordet-Gengou agar in which the NaCl has been replaced with $MgSO_4$ (Lacey 1951). In addition, *B. pertussis* has been shown to undergo freely reversible antigenic modulation in which there is switching between the virulent form (X mode) and the avirulent (C mode) in response to the concentration and type of salts, pH, temperature, and presence of nicotinic acid during the growth of the organisms (Lacey 1960, Pusztai and Joo 1967). In the virulent form, *B. pertussis* expresses various biologically active components such as pertussis toxin, dermonecrotic toxin, adenylate cyclase, haemolysin, filamentous haemagglutinin, agglutinogens, and certain outer membrane proteins that are associated with the virulence of the organism. Weiss *et al.* (1983) found that extracellular adenylate cyclase, haemolysin, and pertussis toxin are factors that contribute to the virulence of the organisms in an infant mouse model system and hypothesized that these virulence determinants as well as other virulence factors, may be positively controlled by a trans-acting *vir* gene product. Not only does the *vir* gene product control the phase variation but the *vir* gene being sensitive to the environmental stimuli controls antigenic modulation (Weiss and Falkow 1984). The triggerring factor(s) that control(s) the expression of the *vir* gene has not been determined. Nicosia and Rappuoli (1987) showed by analysis of a *Tn* 5 mutant of *B.*

*pertussis* (Weiss *et al.* 1983) that the product of the *vir* locus was required for the transcription of the pertussis toxin operon.

Peppler and Schrumpf (1984) reported reversible phenotypic modulation in strains of *B. bronchiseptica* in response to growth on media other than Bordet-Gengou or blood agar. Collings and Rutter (1985) reported the *in vivo* antigenic modulation of *B. bronchiseptica* possibly caused by the immune response of the host to phase I antigens. The molecular mechanisms of control of phenotypic and antigenic modulation in *B. bronchiseptica* are unknown. Lax (1985) suggested that antigenic modulation exhibited by *B. bronchiseptica* strains in response to different growth conditions both *in vitro* and *in vivo* may in fact, be more important in understanding the pathogenesis of bordetellosis than the phase variation observations. Lax (1985) hypothesized that the phase variants seen after *in vitro* phase variation were those organisms which had switched by antigenic modulation control mechanisms and had become trapped through a low rate of mutation and then were selected for by *in vitro* culture techniques.

**1.4 Influence of Bacteriophage on Expression of Virulence-Associated Factors.** Bacteriophage have been reportedly isolated from strains of *B. pertussis* (Mebel *et al.* 1980, 1981) and *B. bronchiseptica* (Rauch and Pickett 1961). Of particular interest to the study of virulence expression within the *Bordetella* species are the findings of Mebel *et al.* which have indicated that the virulence of both *B. pertussis* and *B. parapertussis* strains can be influenced by bacteriophage isolated from heterologous *B. pertussis* strains. The bacteriophage isolated from *B. bronchiseptica* strains have not been examined as possible contributors to the virulence of the organisms that carry them. A review of bacteriophage biology pertinent to the

discussion of the influence of bacteriophage on the virulence of the *Bordetella* species is provided in sections 1.5. to 1.5.2.3.

**1.4.1 Bacteriophage of *B. pertussis*.** Bacteriophage have been isolated from various strains of *B. pertussis* which have been shown to alter the expression of virulence-associated factors of both *B. parapertussis* and heterologous strains of *B. pertussis* (Mebel *et al.* 1980). Bacteriophage were released, both spontaneously and by mitomycin-C induction from laboratory passaged strains and clinical isolates of *B. pertussis* (Mebel *et al.* 1981). Host range determinations revealed the bacteriophage produced both clear and turbid plaques on all strains of *B. parapertussis*, certain strains of *B. bronchiseptica* and certain heterologous strains of *B. pertussis*. Colonies of bacteriophage-resistant *B. pertussis* and *B. parapertussis* with altered colonial morphologies and growth rates were isolated from the plaques. These bacteriophage-resistant colonies, both *B. pertussis* and *B. parapertussis*, were able to produce bacteriophage both spontaneously and through mitomycin-C induction and were considered by the investigators to be lysogenic bacteria. On further characterization of the bacteriophage resistant colonies of *B. pertussis*, several alterations in the phenotype of the host bacteria were observed, presumably under the direct influence of the bacteriophage. The lysogenic strains of *B. pertussis* or variants, grew to form colonies in 24 hours that were urease positive, and appeared to have altered growth rate and biochemical capacity. The bacteria also exhibited changes in their outer membrane components as measured by serology. The variants possessed species-specific agglutinogens for more than one species of *Bordetella*. The variants were able to multiply within mouse spleens for up to ten days (test period) but without splenic atrophy or splenomegaly. One variant resulted in a lower $LD_{50}$, produced higher levels of toxic compounds, produced higher levels of histamine sensitization and was

lethal on intracerebral challenge to mice as compared with the parental strain of *B. pertussis*. Both the parental strain and the strain from which the bacteriophage was originally isolated produced a lower level of toxic products including histamine sensitizing factor and was not lethal on intracerebral challenge of mice. The variants also had a decreased sensitivity to antibiotics. Other colonies of newly lysogenic *B. pertussis* displayed *B. parapertussis*-like traits as evidenced by alterations in the serotype of the *B. pertussis* to an organism with the serotype similar to *B. parapertussis* (Mebel and Lapaeva 1982). Mebel and Lapaeva (1982) reported toxogenic variants of *B. parapertussis* with manifold combinations of agglutinogens specific for all three *Bordetella* species. A hypothesis was forwarded that *B. parapertussis* was merely a form of *B. pertussis* that had been cured of bacteriophage (Granstrom and Askelof 1982). The genetic relatedness of the two organisms, coupled with the clinical finding of co-isolation of *B. pertussis* and *B. parapertussis* during infection were used to support the theory.

Recent studies, however, do not confirm the switching between *B. pertussis* and *B. parapertussis* . Studies by Musser *et al.* (1986) indicate that although there is a close genetic relatedness between the two organisms as measured by allelic enzyme diversity, the two species have sufficient genetic distance to make the conversion highly unlikely. Arico and Rappuoli (1987) found after comparison of the nucleotide sequence of the pertussis toxin operon present in *B. pertussis* and *B. parapertussis*, that conversion between the two species is unlikely. Paloheimo *et al.* (1987) could not corroborate the initial observations of Mebel and Lapaeva (1982). Paloheimo *et al.* (1987) reported that the bacteriophage-resistant variants considered to be lysogenic *B. parapertussis* by Mebel and Lapaeva were actually persistantly infected cells or pseudolysogens (see sections 1.5.2.1 and 1.5.2.2). These variants showed no

increase in virulence or alteration of agglutinogen pattern. The only difference between the parental strain and the pseudolysogens was an altered lipopolysaccharide (LPS) from the normally smooth type found in *B. parapertussis* to a rough LPS characteristic of *B. pertussis*. Further, Paloheimo *et al.* (1987) suggested that the earlier observations of an altered serotype/agglutinogen pattern were due to auto-agglutination as a result of the rough LPS of the variants. These observations, while addressing the conversion of *B. parapertussis* into *B. pertussis* through lysogenic conversion, do not explain the increase in the virulence observed in "converted" heterologous strains of *B. pertussis*.

**1.4.2 Bacteriophage of *B. bronchiseptica*.** Bacteriophage were also reported isolated from several strains of *B. bronchiseptica* (Rauch and Pickett 1961). Host range determination revealed that the bacteriophage were able to plaque on certain heterologous strains of *B. bronchiseptica* and all strains of *B. parapertussis* but not on any of the strains of *B. pertussis*. The further characterization of the bacteriophage was not done as the investigators were trying to probe the relatedness of *B. bronchiseptica* to *B. pertussis* and *B. parapertussis* and also develop a bacteriophage typing scheme to distinguish strains of *B. bronchiseptica*. The ability of *B. bronchiseptica* bacteriophage to lyse cultures of *B. parapertussis* suggested a similar, if not identical, receptor site on both organisms and that the organisms were similar enough to belong to the same genus. A practical bacteriophage typing scheme could not be established from the collection of bacteriophage due to the instability of the bacteriophage and the relative insensitivity of some strains to the bacteriophage.

The incidence of bacteriophage within the genus *Bordetella* and the possible contribution of the bacteriophage to the expression of phenotype of the *Bordetella* species are intriguing avenues for further investigation. The bacteriophage isolated

from the *Bordetella* species to date are poorly characterized and there is some confusion as to the actual relationship, either lyogeny or pseudolysogeny, between the bacteriophage and the host bacterial cell (see section 1.5.2.1 and 1.5.2.2). Further investigation of the *Bordetella* bacteriophage requires characterization of the bacteriophage both with respect to properties of the bacteriophage particles and the relationship of the bacteriophage to the host *Bordetella* cells.

**1.5 Bacteriophage.** To understand the relationship between *Bordetella* bacteriophage and the host bacterial cell especially regarding expression of virulence determinants or possible conversions, it is important to define the current understanding of bacteriophage biology. Bacteriophage are structurally and functionally adapted as obligate parasites of bacterial cells. Structurally, a bacteriophage consists of a nucleic acid core of either DNA or RNA surrounded by a protein coat which affords protection to the phage nucleic acid. The bacteriophage may or may not possess an envelope. The outermost components of the bacteriophage are responsible for adherence to a suitable host for the further propagation of the bacteriophage. The bacteriophage particles are functionally metabolically inert and therefore totally dependent upon the host cell for all biosynthetic and replicative functions. The means by which the bacteriophage replicate have profound effects upon the bacterial host.

**1.5.1 Propagative Strategies of Bacteriophage.** The bacteriophage have been divided into two seemingly mutually exclusive classes, virulent and temperate phages, by virtue of their propagative strategies.

**1.5.1.1 Lytic Cycle.** Virulent bacteriophage propagate through the lytic cycle which involves the injection of the phage nucleic acid into the host bacterial cell and subverting the function of the host cell to the production of maximal numbers of bacteriophage progeny. The lytic cycle, as the term suggests, results in the death

through lysis of the host bacterial cell with concomitant dispersal of maximal numbers of phage particles.

**1.5.1.2 Lysogenic Cycle** In contrast, temperate bacteriophage have the option of propagation through the lysogenic cycle. After injection of the temperate bacteriophage nucleic acid into the host cell, the bacteriophage genome can integrate into the bacterial chromosome and enter into the prophage state where it replicates in synchrony with the host chromosome. The prophage retains the ability to excise from the bacterial genome and enter the lytic cycle either spontaneously or under the influence of inducing agents such as ultraviolet light, mustard gas, mitomycin-c, or hydrogen peroxide. Not all temperate bacteriophage can be induced by inducing agents (Bradley 1967, Barksdale and Arden 1974).

**1.5.2 Relationships of Bacteriophage and Bacterial Host Cells.** Bacteria infected with a lytic bacteriophage will be lysed but bacterial populations infected with a lytic bacteriophage may enter into an equilibrium state with the bacteriophage (see section 1.5.2.3). However, the bacterial cell that carries a prophage is, by definition, a lysogenic bacterium and as such, possesses certain properties. Lysogeny is a heritable trait conferring lysogenic bacteria with immunity against infection by other bacteriophage particles of similar nature and the capacity to produce bacteriophage.

**1.5.2.1 Lysogenic Conversion.** In certain cases, lysogeny can produce phenotypic modification in the lysogenic host either due to the expression of the prophage genome or by switching on or off the bacterial genes. These bacteriophage-determined phenotypic modifications, termed lysogenic conversions have been well studied in various bacterial strains. Medically important examples of lysogenic conversion include toxigenic strains of *Corynebacterium diphtheriae* (Groman 1955 , Groman and Eaton 1955 , Laird and Groman 1976 , Costa *et al.* 1981), Group A

Streptococci (Zabriskie 1964 , Johnson *et al.* 1980., Weeks and Feretti 1984.; Johnson *et al.* 1986), and enteropathogenic strains of *Escherichia coli* (EPEC) (O'Brien *et al.* 1984 , Scotland *et al.* 1985., Strockbine *et al.* 1986) which are converted to the toxigenic phenotype through expression of specific prophage genes in the host bacteria.

**1.5.2.2 Pseudo-Lysogeny.** The lysogenic state possibly resulting in lysogenic conversion, must be distinguished from the carrier state or pseudolysogeny and its possible ensuing bacteriophage conversion. Pseudolysogeny is a persistant lytic bacteriophage infection in which the bacteriophage can infect only a fraction of the bacterial population. The bacteriophage involved in the carrier state are lytic bacteriophage, either obligately lytic, virulent mutants or clear-plaque mutants of temperate bacteriophage (Barksdale and Arden 1974). The lytic infection is kept in check by conditions within the bacterial population. One means of establishing pseudolysogeny is by limiting the number of available receptors on each bacterial cell, either through a process of natural selection or by the action of endolysin. Bacteriophage-sensitive bacteria give rise to resistant mutants having no bacteriophage receptors at a rate as high as $10^{-3}$ to $10^{-4}$ (Baker *et al.* 1949) which are selected for by being able to survive the lytic infection. These resistant mutants have an altered phenotype due to the presence of the bacteriophage but the changes are not directed by the bacteriophage. In other systems such as T7 infection of *Shigella dysenteriae*, infected cells produced a receptor-destroying endolysin which is produced by resistant cells without phage receptors (Li *et al.* 1961). The resulting population was comprised of two sub-populations, one being bacteriophage-sensitive and producing bacteriophage and the other rendered resistant to bacteriophage infection by the action of endolysin. Again, the bacterial phenotype is altered due to the presence of the

bacteriophage with the host bacterium but the changes are not directed by the bacteriophage.

**1.5.2.3 Bacteriophage Conversions.** Pseudolysogeny may manifest itself in modifications of the bacterial phenotype. Such phenotypic modifications are bacteriophage conversions rather than lysogenic conversions. The modification involves only apparent rather than actual genetic changes in the host cell as a result of bacteriophage infection (Barksdale and Arden 1974). Li *et al.* (1961) reported lactose-negative strains of *Shigella dysenteriae* which normally grew as clear colonies on MacConkey's plates would convert to red, apparently lactose-positive colonies when carrying the bacteriophage T7. A T7 endolysin changed the permeability of the bacterial cell, allowing leakage of macromolecules, in this case ß-galactosidase, into the medium to produce the apparent phenotypic modification. The modified phenotype of the *S. dysenteriae*-T7 clones was stable even after fifty-seven serial single colony isolations. Many characteristics of the pseudolysogenic and lysogenic strains are very similar. In both conditions the host bacterium is resistant to infection by other related bacteriophage, can produce bacteriophage and may express stable heritable phenotypic modification. Demonstration of the bacteriophage genome integrated into the bacterial chromosome is the method to establish a true lysogenic state. The distinction between virulent and temperate bacteriophage and whether they produce a lytic, lysogenic, or pseudolysogenic state with the bacterial host is further muddied by the fact that many bacteriophage are capable of both propagative cycles depending upon the bacterial strain they infect. The terms can thus be used as a description of a bacteriophage-host system rather than as a means of classification (Bradley 1967).

**1.6 Bacteriophage Classification.** This thesis describes the isolation and characterization of a new bacteriophage from *B. bronchiseptica*. It was necessary to

ascertain which properties of the bacteriophage should be reported to allow meaningful comparisons with other bacteriophage. A classification system is required to provide a means of cataloguing the vast numbers of reported bacteriophage isolates as well as a framework within which the systematic reporting of novel bacteriophage isolates can proceed. Taxonomic grouping, defined by salient properties of the bacteriophage, determine if in fact a "new" bacteriophage has been isolated and also facilitates comparitve study of the "new" isolate with other bacteriophage. A system of nomenclature is inherent in taxonomy and would standardize the naming of bacteriophage (Adams 1959). To date, the classification system for bacteriophage is still in a rudimentary state, although much advancement has been made in recent years (Ackermann 1987).

**1.6.1 Taxonomy of Bacteriophage.** The foundations of the present taxonomic system for bacteriophage are gleaned from the various attempts to classify bacteriophage. D'Herelle considered all bacteriophage to be a single species, *Protobios bacteriophagus*, with extreme powers of variation (Adams 1959). As more bacteriophage were isolated and studied, it became clear that the single species concept was premature. Bacteriophage were found to be a heterogeneous population extremely stable upon serial passage with high host specificity, all properties unreconsilable with the proposed powers of adaptive variation. Systematic bacteriophage classification was first devised for groups of bacteriophage specifically attacking a group of bacteria. Bacteriophage classification was based on distinct serological groupings but other characteristics such as plaque morphology, resistance patterns of bacteriophage-resistant host cells, particle size and sensitivity to inactivation by urea, chloroform, heat, and ether were correlated with the serological specifities (Burnet 1933, Evans and Sockrider 1942, Adams 1952, Friedman and Cowles 1953, Adams and Wade

1954, Wilkowske *et al.* 1954, Adams and Wade 1955). Holmes (1948) was the first to propose an extensive taxonomic system encompassing all known viruses. Bacteriophage, animal viruses and plant viruses were classified as suborders in the order Virales. Bacteriophage were placed in a single genus "phagus" and forty-six species based primarily on host range and symptoms of the disease. This system was never implemented by researchers in the field, mainly because of the reliance on host-related rather than virion-specific characteristics to classify the viruses. Adams (1953) proposed an extensive taxonomic system for bacteriophage based on more virion-specific characteristics and leaving the relationship between bacteriophage and plant and animal viruses for study at a later time. "Species" as defined by Adams was based on distict serological groupings, the size and morphology of the virion, unique chemical composition, latent period, susceptibility to inactivation, and distinctive physiological properties (such as calcium requirement for bacteriophage adsorption). He proposed that taxonomic divisions above the level of species could be determined by electron microscopy and that the Linnaean binomial nomenclature not be implemented until genus and species assignments had been determined. The propsals of Lwoff and Tournier (1962) established the criteria by which all viruses, including bacteriophage could be classified. The basis of the classification, four features called the "essential integrants" of the virion are as follows: (1) genetic material (DNA or RNA); (2) symmetry of the virus: helical, cubical, or binal (refering to viruses with head and tail); (3) nucleocapsid: naked or enveloped; (4) quantitative data: diameter of the nucleocapsid for the virion with helical symmetry; number of capsomeres for the virion with cubic symmetry. The taxonomic system was hierarchical with arbitrary assignment of the lowest taxonomic group as family. No phylogenetic relationships were established and there was room for subdivsions of genus and species. The

system was the most workable of its predecessors with the groups currently known as Microviridae, Leviviridae, and the Inovirus genus still in use in the present classification system (Ackermann 1987). Lwoff and Tournier (1962) also grouped all tailed bacteriophage in one order Urovirales.

Bacteriophage taxonomy has a unique problem in that 95% of all bacteriophage were tailed and were in the order Urovirales (Ackermann 1987). Bradley (1965) proposed further distinction of bacteriophage by morphological details of the tail structure. Of significance was the separation of Urovirales into three smaller groups characterized by having contractile tails; long, noncontractile tails and short, non-contractile tails. Bacteriophage morphotype as described by Bradley is critical information for the classification of bacteriophage in the presently accepted taxonomic scheme for bacteriophage.

**1.6.2 Present Criteria for Phage Taxonomy.** In 1966, the International Committee of Taxonomy of Viruses (I.C.T.V.) was founded. Sub-committees were formed for the specific classification of viruses infecting vertebrates, invertebrates, fungi, and bacteria but all use the same basic rules of virus classification. The ICTV use every known property of the virus for the classification and has no hierarchy of criteria. The criteria, considered with equal weight by the I.C.T.V. for the classification of bacteriophage are (1) nucleic acid: nature, mass, composition; (2) virion: symmetry, dimensions, mass, gross composition; (3) antigenic relationships, host range, and resistance against environment (chiefly ether, chloroform, heat). New criteria may be added as DNA-DNA hybridization and DNA sequencing are developed (Ackermann 1987). There are ten families and eight genera approved by the I.C.T.V. (Table 3).

**Table 3**: Basic characteristics and frequency of major phage groups

| Shape | Nucleic acid | Family | Genus | Particulars | Members |
|---|---|---|---|---|---|
| Tailed | DNA, 2, L | Myoviridae | ------ | Tail,contractile | 811 |
| | | Siphoviridae | ------ | Tail long, non-contractile | 1469 |
| | | Podoviridae | ------ | Short tail | 441 |
| Cubic | DNA, 1, C | Microviridae | Microvirus | Conspicous capsomers | 30 |
| | 2, C, S | Corticoviridae | Corticovirus | Complex capsid,lipids | 2? |
| | 2, L | Tectiviridae | Tectivirus | Double-capsid, pseudo-tail, lipids | 14 |
| | RNA, 1, L | Leviviridae | Levivirus | ------ | 41? |
| | 2, L, seg | Cystoviridae | Cystovirus | Envelope, lipids | 1 |
| Filamentous | DNA, 1, C | Inoviridae | Inovirus | Long rods | 25 |
| | | | Plectovirus | Short rods | 15 |
| | 2, L | F3 Group | -------- | Envelope, lipids | 3 |
| Pleomorphic | DNA, 2, C, S | Plasmaviridae | Plasmavirus | Envelope, lipids, no capsid | 3 |

Abbreviations : C = circular ; L = linear ; S = superhelical ; seg = segmented ; 1 = single-stranded; 2 = double-stranded ; ---- = none.

(Adapted from Ackermann 1987)

**1.7 Scope of Project** The goal of this project was to characterize a bacteriophage which was spontaneously released from a virulent strain of *Bordetella bronchiseptica*. The characterization followed the guidelines established by the I.C.T.V. for the taxonomic classification of bacteriophage as well as additional information in the form of a restriction enzyme map of the bacteriophage nucleic acid. In order to determine if the bacteriophage was present within other strains of *B. bronchiseptica* or the other *Bordetella* species, the bacteriophage nucleic acid was radiolabelled and used to probe strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* through DNA hybridization techniques. The actual relationship between the host bacteria and the bacteriophage, either lysogenic or pseudolysogenic, was explored.

# 2.0 Materials and Methods

## 2.1 Bacterial Strains:

The strain *Bordetella bronchiseptica* AG1-L used was sent to Dr. M. S. Peppler (Department of Medical Microbiology and Infectious Diseases, University of Alberta) by Dr. Hilton Klein (Whittaker M. A. Bioproducts, Walkersville, Maryland) from an outbreak of lethal bordetellosis in a green monkey (*Cercopithecus aethiops*) colony. Previous work had shown that *B. bronchiseptica* AG1-L spontaneously produced bacteriophage and was not inducible by irradiation with ultraviolet light. Host range determination of the bacteriophage had revealed that it plaqued on three of six *B. bronchiseptica* strains and on all fifteen strains of *B. parapertussis* tested. There were no plaques formed on any of the five strains of *B. pertussis* tested (M. S. Peppler, unpublished data).

The indicator organism used was *Bordetella parapertussis* 17903 obtained from the Michigan Department of Health, Grand Rapids, Michigan.

The following strains of *Bordetella pertussis* , *Bordetella parapertussis* and *Bordetella bronchiseptica* were used: (Peppler 1982, Peppler and Schrumpf 1984, Musser *et al.* 1986 )

| Strains | Source |
|---|---|
| *B. pertussis* | |
| $3779^{+}$ | J. J. Munoz, Rocky Mountain Laboratories, |
| $3779^{-}$ | Hamilton, Montana |

| | |
|---|---|
| BB103 | C. R. Manclark, Food and Drug Administration Bureau of Biologics, Bethesda, Md. |
| 2753 | the mouse challenge strain *B. pertussis* 18323 of P. Kendrick obtained through Merck Sharpe & Dohme |
| $SAK^+$ | Kachiko Sekiya, Kitasato University, Tokyo, |
| $SAK^-$ | Japan |

*B. parapertussis*

| | |
|---|---|
| 504 | H.-W. Ackermann, Laval University, Quebec |
| PL-1 | Provincial Laboratory, Edmonton , Alberta |
| 17903 | Michigan Department of Health, Grand Rapids, MI. |

*B. bronchiseptica*

| | |
|---|---|
| Rab-10 | J. J. Munoz, as above |
| 214 | J. J. Munoz |
| AG1-L | H. Klein |
| AG9-L | H. Klein |
| AG11-L | H. Klein |
| AG11-H | H. Klein |
| 110-H | D.A. Bemis, University of Tennessee, Knoxville |
| Rat-1 | D.A. Bemis |
| BTS | D.A. Bemis |
| 501 | D.A. Bemis |

| | |
|---|---|
| 17640-SAC | D.A. Bemis |
| Columbus | D.A. Bemis |
| 87 | D.A. Bemis |
| Ft. Collins | D.A. Bemis |

The coliphages T4 and λ were propagated on *Escherichia coli* strains 364 and 176 respectively. The coliphages and their respective hosts were kindly provided by Dr. D. E. Taylor (Department of Medical Microbiology and Infectious Diseases, University of Alberta).

**2.2 Media :**

**2.2.1. Bordet-Gengou Agar (BGA) :** (see appendix)

(Peppler, 1982)

**2.2.2. Cyclodextrin Solid Medium (CSM) :** (see appendix)

(Aoyama *et al.* 1986)

**2.2.3. Trypticase Soy Broth (TSB) :**

Trypticase soy broth was prepared by dissolving 30 g of trypticase soy broth powder (Difco) in 1 liter of double distilled water. The broth was dispensed into Wheaton bottles in 100 ml portions and autoclaved for 20 minutes.

**2.2.4. Trypticase Soy Agar (TSA) :**

Trypticase soy agar was prepared by dissolving 30 g of trypticase soy broth powder and 10 g of Nobel agar (Difco) into 1 liter of double distilled water. The agar

was autoclaved for 20 minutes. The agar was transferred to a 56°C water bath and poured when the agar had reached 56°C.

**2.2.5. Stainer-Scholte Broth (SSB) :** (see appendix)

(Stainer and Scholte 1971)

**2.2.6. Stainer-Scholte Agar (SSA) :** (see appendix)

(Peppler 1982)

**2.2.7. Luria-Bertani Medium (LB) :**

Luria-Bertani medium was prepared by dissolving 10 g of Bacto-tryptone, 10 g of NaCl, and 5 g of Bacto-yeast extract to 1 liter of double distilled water and adjusting the pH to pH 7.5 with 10N NaOH. The broth was dispensed into Wheaton bottles in 100 ml portions and autoclaved for 20 minutes.

**2.2.8. Luria-Bertani Agar (LA) :**

Luria-Bertani agar was prepared by adding 10 g of Nobel agar (Difco) to 1 liter of LB and autoclaving for 20 minutes. The agar was cooled to 56°C and poured.

**2.2.9. Sloppy agars**

**2.2.9.1 PBS-Sloppy-agar** : PBS-sloppy agar was prepared by dissolving 0.6 g of Nobel agar (Difco) into 100.0 ml of phosphate-buffered saline (PBS, see section 2.3.2) and autoclaving for 15 minutes. The sloppy agar was dispensed soon after removal from the autoclave.

**2.2.9.2. Luria-Bertani Sloppy-agar** : Luria-Bertani sloppy agar was prepared by dissolving 0.6 g of Nobel agar (Difco) into 100 ml of LB and autoclaving for 15 minutes. The sloppy agar was dispensed soon after removal from the autoclave.

**2.2.10 Storage of the Various Media.** All autoclaving was done at 121°C and at a pressure of 18 psi. All agars (with the exception of the sloppy agars) were dispensed in 15-20 ml portions into 15 x 100 mm plastic petri dishes. After pouring, the plates were allowed to dry at room temperature for 12-16 hours before storing at 4°C in the plastic bags which had originally contained the unused petri dishes. The agar was used within two weeks. The sloppy agars were dispensed in 2.5 ml portions into sterile 13 x 100 mm screw-capped tubes and stored at 4°C until required. The sloppy agars were used within two months of preparation. The broth media were stored in glass Wheaton bottles at room temperature and used within two months.

## 2.3 Chemicals and Reagents:

**Buffers:**

**2.3.1 SDS-PAGE (Laemmli) Running Buffer** (Laemmli 1970)

The following was dissolved in 4.5 liters of Milli-Q™ water :

| | |
|---|---|
| 25 mM Tris (Sigma 7-9) | 15.125 g |
| 191.7 mM Glycine (Sigma) | 72.0 g |
| SDS (BDH or Bio-RAD) | 5.0 g |

The volume was adjusted to 5 liters and stored at room temperature until use.

### 2.3.2 Phosphate-Buffered Saline (PBS)

The following salts were dissolved in 0.8 liter of distilled water :

| | |
|---|---|
| 54 mM $Na_2HPO_4$ (anhydrous) | 7.65 g |
| 13 mM $KH_2PO_4$ (anhydrous) | 1.74 g |
| 73 mM NaCl | 4.24 g |

The volume was adjusted to 1 liter and the PBS dispenced into 200 ml aliquots, autoclaved to sterilize and stored at room temperature.

### 2.3.3 Lambda diluent (Schleif and Wensink 1981)

Working solution is 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$

Concentrated solutions (100 x) are 1 M Tris-HCl ( pH 7.5) and1 M $MgCl_2$.

Concentrated Lambda diluent solutions were filter sterilized through a 0.22 µm Nalgene filter and stored at room temperature. Working solutions were made up from the concentrated stocks as necessary.

### 2.3.4 Tris-Acetate (TA) buffer (Maniatis *et al.* 1982)

Working solution is 0.004 M Tris-acetate, 0.001 M EDTA.

Concentrated Stock Solution (10 x)

Per liter :

| | |
|---|---|
| Tris base | 48.4 g |
| glacial acetic acid | 11.42 ml |
| 0.5 M EDTA ( pH 8.0) | 20 ml |

One liter volumes of stock TA buffer (10x) were prepared and stored at room temperature.

**2.3.5 Tris-Borate (TB) buffer** ( Maniatis *et al.* 1982)

Working solution is 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA

Concentrated Stock Solution (10x)

Per liter :

| | |
|---|---|
| Tris base | 108.0 g |
| boric acid | 55.0 g |
| 0.5 M EDTA (pH 8.0) | 40.0 ml |

One liter volumes of stock TB buffer (10x) were prepared and stored at room temperature.

**2.3.6 Agarose Gel-loading buffer** (Maniatis *et al.* 1982)

A 10 x buffer consisting of 0.25 % bromophenol blue, 0.25 % xylene cyanol, 25 % Ficoll (type 400) in water. The buffer was stored at room temperature.

**2.3.7 Ethidium Bromide Buffer** (Dr. A. R. Morgan, Department of Biochemistry, University of Alberta)

A solution of the following composition was prepared for the fluorometric readings of the DNA samples :

5mM Tris-HCl (pH 8.0)

0.5 mM EDTA

0.5 µg ethidium bromide

**2.3.8 SSPE** (20x) (Maniatis *et al.* 1982)

A stock solution was prepared by dissolving 174 g of NaCl, 27.6 g of $NaH_2PO_4 \cdot H_2O$, and 7.4 g of EDTA in 800 ml of $H_2O$. The pH was adjusted to 7.4 with NaOH (~ 6.5 ml of a 10 N solution). The volume was adjusted to 1 liter, dispensed into aliquots and sterilized by autoclaving.

**2.3.9 SSC** (20x) (Maniatis *et al.* 1982)

A stock solution was prepared by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml $H_2O$. The pH was adjusted to 7.0 with a few drops of 10 N solution of NaOH. The volume was adjusted to 1 liter, dispensed into aliquots and autoclaved to sterilize.

**Staining reagents**

**2.3.10 Coomassie brilliant blue R-250 stain** (Peppler 1982)

The stain consisted of 0.2% Coomassie brilliant blue R-250 (BIO-RAD) dissolved in a 7% acetic acid-25% isopropanol. The destaining solution was 7% acetic acid-25% isopropanol. The gels were washed in a 7% acetic acid solution as a final destain and storage solutions.

**2.3.11 Silver stain reagents** (Tsai and Frasch 1982)

Reagent grade ammonium hydroxide solution (29% wt./vol) and stored at room temperature in the manufacturers glass bottle.

Silver nitrate solution was made to 20% wt./vol., filtered through a 0.22 µm Amicon filter and stored at 4°C in a brown bottle.

The developer was made by adding 0.5 ml of a 37% formalin solution and 50 mg of citric acid to 1 liter of distilled water. This solution was stored at room temperature.

**2.3.12 Ethidium bromide stain** (Maniatis *et al.* 1982)

A stock solution of 10 mg / ml of ethidium bromide was prepared by adding 1 g of ethidium bromide to 100 ml of $H_2O$. The solution was mixed on a magnetic stirrer for several hours, the bottle wrapped in tin foil, and stored at 4°C.

The stain for the agarose gels was prepared by adding 50 µl of the stock ethidium bromide solution to a tray containing ~ 100 ml of distilled water

**Enzymes**

**2.3.13 Restriction enzymes**

All restriction enzymes were supplied by Boerhringer-Mannheim or Bethesda Research Laboratories except the enzymes listed below with their respective supplier.

*Afl* II (Amersham )

*Apa* I (Pharmacia)

*Bssh* II (New England Bio Labs)

*Bst* I (Pharmacia)

*Mlu* I (Pharmacia)

*Not* I (New England Bio Labs)

*Sac* I (Pharacia)

All restriction enzymes were used in accordance with the manufacturers instructions.

**2.3.14 Proteinase K** (Boehringer-Mannheim) was made up to a stock solution of 1mg/ml in distilled water and aliquots stored at -20°C.

**2.3.15 RNase** (Boehringer-Mannheim) was made up to a stock solution of 5 mg/ml, boiled for 5 minutes to destroy any DNase activity, dispensed into 1 ml aliquots and stored at -20°C.

**Solutions**

**2.3.16 Equilibrated phenol** (Maniatis *et al.* 1982)

Liquified phenol was redistilled at 160°C. The redistilled phenol was extracted several times with an equal volume of buffer until the pH of the aqueous phase was > 7.6. The buffers used were 1.0 M Tris (pH 8.0) followed by 0.1 M Tris (pH 8.0) and 0.2% ß-mercaptoethanol. The equilibrated phenol solution was stored at 4°C for periods of up to 1 month or at -20°C for up to 6 months.

**2.3.17 Isoamyl alcohol-chloroform** (Maniatis *et al.* 1982)

A mixture of chloroform and isoamyl alcohol ( 24:1 v/v) was prepared and stored at room temperature.

**2.3.18 Ether saturated with water** (Maniatis *et al.* 1982)

A mixture of an equal volume of anhydrous ethyl ether and distilled water was added to a screw-capped bottle. The mixture was shaken and then stored in a hood at room temperature.

**2.3.19 3 M sodium acetate** (Maniatis *et al.* 1982)

The solution was prepared by dissolving 408.1 g of sodium acetate·$3H_2O$ in 800 ml of $H_20$. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 liter, dispensed into aliquots and sterilized by autoclaving.

**2.3.20 5 M EDTA** (Maniatis *et al.* 1982)

The solution was prepared by dissolving 186.1 g of disodium ethylene-diaminetetraacetate·$2H_2O$ in 800 ml $H_2O$. The solution was vigorously mixed on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH (~20 g of NaOH pellets). The solution was dispensed into aliquots and autoclaved to sterilize.

**2.3.21 10% SDS** (Maniatis *et al.* 1982)

The solution was prepared by dissolving 100g of electrophoresis grade sodium dodecyl sulfate in 900 ml $H_2O$ and heating to 68°C. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The solution was dispensed into aliquots. No sterilization was necessary.

**2.3.22 1 M Tris** (Maniatis *et al.* 1982)

The solution was prepared by adding 121.1 g of Tris base to 800 ml $H_2O$ and adjusting the pH to the desired value by adding concentrated HCl. (pH 7.4 add ~ 70 ml HCl, pH 7.6 add ~ 60 ml HCl, pH 8.0 add ~ 42 ml HCl )

The solution was allowed to cool to room temperature. The volume was adjusted to 1 liter, dispensed into aliquots and autoclaved to sterilize.

**2.3.23 10% TCA** (Maniatis *et al.* 1982)

A stock solution (100% wt./vol.) was prepared by adding 227 ml of $H_2O$ to 500 g of trichloroacetic acid. A 1 in 10 dilution was done to prepare the 10% TCA working solution.

**2.3.24 Laemmli digestion mix** (Laemmli 1970)

The Laemmli digestion mix was prepared by mixing the following materials:

| | |
|---|---|
| 10% SDS (BDH) | 2.0 ml |
| Dithiothreitol (DTT) | 0.078 g |
| -or-(ß-mercaptoethanol) | (0.4 ml) |
| glycerol (glycerin) | 1.0 ml |
| 1.0 M Tris pH 6.8 | 0.625 ml |
| Milli-Q™ water | 6.0 ml |
| Saturated bromophenol blue (in water) | 0.075 ml |
| | 10.025 ml |

The Laemmli digestion mix was stored at room temperature.

**2.3.25 SDS-PAGE reagents.** ( Laemmli 1970, Peppler 1982)

Separating gels consisted of either 12.5% or 14% wt. / vol. total acrylamide:

| | 12.5 % or 14 % | |
|---|---|---|
| Acrylamide:BIS (30:0.8) | 12.5 or | 14 ml |
| 1.875 M Tris pH 8.8 | 6.0 ml | 6.0 ml |
| 0.2 M disodium EDTA | 0.3 ml | 0.3 ml |
| Miili-Q™ water | 10.9 ml | 9.4 ml |
| TEMED | 0.015 ml | 0.015 ml |
| 10% Ammonium persulfate | 0.3 ml | 0.3 ml |
| | 30.015 ml | 30.015 ml |

The TEMED was added prior to addition of the 10% ammonium persulfate. The ammonium persulfate solution was prepared freshly just prior to use and stored on ice. A disposable pipette was used to deliver 3.5 ml of the separating gel without delay to the mini-gel apparatus. A shallow layer of Milli-Q™ water was overlaid on the polymerizing gel. The gel was allowed to set for 15 minutes prior to pouring the stacking gel.

Stacking gel consisted of 5% wt./vol. total acrylamide

| | |
|---|---|
| Acrylamide:BIS (30:0.8) | 2.50 ml |
| 1.0 M Tris, pH 6.8 | 1.88 ml |
| 0.2 M disodium EDTA | 0.15 ml |
| Milli-Q™ water | 10.3 ml |
| TEMED | 0.008 ml |
| 10% ammonium persulfate | 0.15 ml |
| | 15.0 ml |

The stacking gel was added to the top of the separating gel with a Pastuer pipette and allowed to polymerize for a minimum of 45 minutes prior to use.

**2.3.26 Protein molecular weight standards** The low molecular weight standards used in the protein profile determination were supplied by BIO-RAD Laboratories.

**2.3.27 Iodo-Gen™** was supplied by the Pierce Chemical Company. The chemical name of the compound is 1,3,4,6-tetrachloro-3α, 6α - diphenylglycouril.

**2.3.28 Anprolene™**, used for gas sterilization, was supplied by H. W. Andersen Products, Inc., 221 South Street, Oyster Bay, New York 11771 U. S. A.

**2.3.29 Geneclean ™** kit was supplied by Bio 101 Inc. P.O. Box 2284 La Jolla California USA 92038-2284.

**2.3.30 Preparation of G75 column** (Maniatis *et al.* 1982)
Thirty grams of Sephadex G-75 (BIO-RAD) was slowly added to 250 ml of distilled water in a 500-ml flask. The flask was autoclaved for 15 minutes at 18 psi then cooled to room temperature. The distilled water was decanted and the beads resuspended in an equal volume of TE buffer (pH 8.0). The G-75 slurry was poured into a suitable sized column and packed under vacuum to

remove trapped air bubbles. The column was washed in several volumes of TE (pH 8.0) before use.

**2.3.31 The cesium chloride solutions** (Handbook of Biochemistry and Molecular Biology Physical and Chemical Data, 3rd edition, volume I, Fasman, G. D. (ed) p 419-23, CRC Press, Boca Raton, Fl, 1982)

The solutions used in the step gradients were prepared by dissolving the solid cesium chloride (99.7% Terochem Laboratories Ltd.) in approximately 100 ml of Milli-Q™ water. The density of the solution was determined by measuring the refractive index of the solution using a refractometer (Fisher) and using standard tables to convert the refractive indices to density values for the solutions. The stock solutions had refractive indices of 1.3627, 1.3725, and 1.3820 corresponding to densities of 1.30166, 1.40809, and 1.51126 at 25°C respectively. The stock solutions were stored at 4°C.

**2.3.32 Diphenylamine Reagent** (Burton 1956)

Diphenylamine reagent was prepared by dissolving 1.5 g of diphenylamine (Fisher Scientific Co.) in 100 ml of acetic acid and adding 1.5 ml of concentrated sulfuric acid. The reagent was stored in the dark. Just prior to use 0.1 ml of aqueous acetaldehyde (16 mg/ml) was added to each 20 ml volume of reagent used.

**2.4. Isolation of Bacteriophage L1:** ( Adams 1959)

*B. bronchiseptica* AGL-1 was grown on Bordet-Gengou agar for 18-24 hours at 37°C in a humidified incubator. A swab was used to a transfer the bacterial growth

to a sterile 13 x 100 mm test tube containing trypticase soy broth. The resulting bacterial suspension was adjusted to an optical density at 540 nm wavelenghth to approximately 0.4. A 0.5 ml aliquot of the adjusted bacterial suspension was added to a 100 ml Erlenmeyer flask containing 20 ml of trypticase soy broth and incubated at 37°C in a shaking incubator (New Brunswick Scientific Co. Model No. 761003) at approximately 100 rpm (a setting of 4) for 5-6 hours. The contents of the flask were transferred to a 20 ml Corex tube and stored at 4°C overnight. The tube was centrifuged at 8 000 x g for 10 minutes to pellet the bacterial debris. The supernatant was filter sterilized through a 0.2 µm Nalgene filter and serially diluted in trypticase soy broth. Ten microliter aliquots were spotted onto sloppy-agar overlay plates of the indicator organism (see section 2.6), *B. parapertussis* 17903, incubated overnight at 37°C and examined for plaque formation. Single plaques were removed from the soft-agar overlay plates with a sterile Pasteur pipette, the agar plug being placed in 1 ml of trypticase soy broth at 4°C overnight. The trypticase soy broth containing the agar plug was serially diluted in trypticase soy broth and spotted in 10 µl aliquots onto indicator plates. The bacteriophage L1 was plaque purified by repeating the above single-plaque isolations three times.

**2.5 Determination of Bacteriophage L1 Titer (Plaque Assay):** (Adams 1959)

Bacteriophage lysates were serially diluted in trypticase soy broth or Lambda diluent. Aliquots (10 µl) were spotted on a single soft-agar lawn of the indicator organism *B. parapertussis* 17903. To maintain the integrity of the spots, the soft-agar plates were kept at room temperature in a biological hood to allow the spots of diluted bacteriophage lysate to soak into the agar. The plates were then incubated at 37°C in a

humidified incubator for 24-30 hours. Plaques were counted with the aid of a colony counter (New Brunswick Scientific Co Model C-110).

**2.6 Preparation of Indicator Plates:** (Adams 1959)

The indicator strain, *B. parapertussis* 17903 was grown up on Bordet-Gengou agar (BGA) for 36-48 hours at 37°C in a humidified incubator. Using a swab, colonies of *B. parapertussis* 17903 were removed from the BGA plate and transferred to a sterile 13 x 100 mm capped test tube containing 4 to 5 ml of trypticase soy broth. The optical density of the bacterial suspension was measured in a spectrophotometer (Coleman Junior® II Spectrophotometer Model 6/20) at a 540 nm wavelength and adjusted to an absorbance reading of 0.20 ± 0.02. A 0.5 ml aliquot of the adjusted bacterial suspension was added to a sterile, screw-capped 13 x 100 mm test tube containing 2.5 ml of molten sloppy agar maintained at 46°C by incubation in a waterbath. The tube was inverted twice to mix and the contents then poured onto trypticase soy agar plates that had been equilibrated to room temperature. The soft-agar overlays were allowed to dry in a biological hood with the fan running for at least two hours prior to use.

**2.7 Growth Curves of the Indicator Strain *B. parapertussis* 17903.**

In order to determine the optical density at which the maximal number of cells at mid-log phase could be obtained and also the doubling time of the organisms, a growth curve of the organisms in TSB was constructed measuring the absorbance and viable counts of the culture over time. A swab was used to transfer the growth from a 24-36 hour BGA culture of *B. parapertussis* 17903 grown at 37°C in a humidified incubator to a sterile 13 x 100 mm screw-capped tube containing approximately 4 ml of TSB.

The bacterial suspension was adjusted to an optical density at 540 nm of 0.20 ± 0.02. A 1 ml sample was transferred to a nephelometry flask containing 100 ml of TSB. The nephelometry flask was placed on a 37°C shaker incubator ( New Brunswick Scientific Co.) and shaken at approximately 100 rpm (a setting of 4). At various time intervals, the optical density of the culture was measured at 540 nm and a 0.1 ml sample of the bacterial culture was aseptically taken and serially diluted in TSB. Fifty microliter samples of the dilutions were plated on both BGA and TSA plates to determine the viable counts.

**2.8 Propagation of Bacteriophage L1:**

**2.8.1. Soft-Agar Overlay Method**: (Adams 1959)

A 24 hour culture of *B. parapertussis* 17903 was grown on Bordet-Gengou agar and a swab used to transfer the growth to a sterile, capped 13 x 100 mm test tube containing 5 ml of trypticase soy broth. The bacterial suspension was adjusted to an optical density at 540 nm wavelength of 0.20-0.22. A 0.4 ml aliquot of the adjusted bacterial suspension was added to a sterile, screw-capped test tube into which 50-100 µl of an bacteriophage L1 lysate was added (usually 50 µl of bacteriophage L1 lysate with a titer of $5 \times 10^7$ to $2 \times 10^8$ PFU / ml). The bacteriophage was allowed to incubate with the bacterial cells at 4°C for a 20 minute period of time. The infected bacteria were then transferred to molten sloppy agar at 46°C, mixed by inversion, and poured onto TSA plates which had been equilibrated to room temperature. A control plate of uninfected *B. parapertussis* 17903 was included each time to check to see if the lawns were healthy. The plates were incubated in the cold room for 30 to 45 minutes and the transferred to a humidified incubator at 37°C and incubated for 24 to 27 hours. Each plate was then flooded with 4.0 ml of Lambda diluent and placed on a shaker at 4°C overnight. The Lambda diluent was removed from the plates using a Pasteur pipette

avoiding aspiration of the soft-agar overlay, transferred to 25 ml Corex tubes and centrifuged at 8 000 x g for 30 minutes. The supernatant was either filter sterilized by passing the lysate through a 0.22 µm Nalgene filter or transferring to a glass bottle containing a few drops of chloroform and stored at 4°C.

**2.8.2. Broth Method**: ( modified from Maniatis *et al.* 1982)

**Preparation of Host Bacteria**: The following technique was used to produce a sufficient quantity of a mid-log phase broth culture of *B. parapertussis* 17903 for infection with bacteriophage L1 required for maximal yields of bacteriophage L1. *Bordetella parapertussis* 17903 was grown on BGA plates for 24 to 36 hours at 37°C in a humidified incubator. The bacterial growth was transferred to a sterile, screw-capped 13 x 100 mm test tube containing 4-5 ml of trypticase soy broth. The absorbance of the bacterial suspension was determined at a wavelenghth of 540 nm in a spectrophotometer and adjusted to a final absorbance of approximately 0.2. A 1 ml aliquot of the adjusted bacterial suspension was transferred to a sterile nephelometry flask containing 100 ml of TSB and fitted with a cotton or foam bung to allow the exchange of air. The nephelometry flask was placed on a shaker incubator at approximately 100 rpm (at a setting of 4) at 37°C and allowed to incubate until the absorbance of the bacterial culture at a 540 nm wavelenghth reached 0.30-0.35 which required approximately 24 to 27 hours. A 20.0 ml aliquot of the bacterial culture was transferred to a sterile, capped Corex tube and centrifuged at 4°C and 8 000 x g to pellet the bacteria. The supernatant was decanted and the Corex tubes inverted on absorbant matting (paper towels covered by Kim Wipes) until the pellets no longer had a layer of fluid on top. The host bacteria were ready for bacteriophage infection.

**Infection of Host Cells with Bacteriophage L1**: To infect the bacterial pellets, 1 ml of bacteriophage L1 lysate of known titer ( ~1-2 x $10^8$ PFU / ml) was

added to the Corex tube and the bacterial pellet resuspended in the lysate. The Corex tube was placed at 4°C for 20 to 30 minutes to allow the adsorption of the bacteriophage to the bacteria. The contents of the Corex tube were then transferred to a 4-liter Erlenmeyer flask containing 500 ml of TSB and placed on a shaker incubator at approximately 100 rpm (at a setting of 4) at 37°C for 24 to 27 hours. Following the incubation, the Erlenmeyer flasks were placed on ice for approximately 30 minutes. The bacterial debris was removed by centrifugation at 2 500 x g for 30 minutes. The resulting supernatant was decanted into sterile Wheaton bottles to which had been added a few drops of chloroform. The bacteriophage lysates were stored at 4°C for several months without loss of infectivity of the bacteriophage lysates.

**2.9 Concentration of Bacteriophage L1 and Purification:** (Maniatis *et al.* 1982)

The bacteriophage stocks, obtained from either the agar or broth method of propagation are divided into 20.0 ml aliquots and placed in Corex tubes. Solid polyethylene glycol was added to each tube to a final concentration of 10% wt / vol. The polyethylene glycol (Sigma 8000) was added in small portions and vortexed vigorously with each addition to ensure no lumps of polyethylene glycol would form. Solid sodium chloride was then added to a concentration of 1 M (approximately 1.2 g). The mixture was vortexed vigorously to dissolve the salt and polyethylene glycol and allowed to sit on ice for 1 hour. The bacteriophage were pelleted by centrifugation at 4°C and 10 800 x g for 1 hour. The supernatant was decanted and the tubes allowed to drain on a matting of Kimwipes and paper towels for several hours at room temperature or overnight at 4°C. Each drained bacteriophage pellet was resuspended in 0.5 ml of TSB, the bacteriophage suspensions pooled, and extracted with an equal volume of

chloroform to remove the polyethylene glycol. The phases were separated by centrifugation at 4°C and 8 000 x g for 15 to 20 minutes. The top phase was removed and maintained either on ice or at 4°C until it could be applied to the cesium chloride step gradients. The step gradients were formed using a peristaltsic pump to sequentially layer CsCl solutions of decreasing density on top of one another in Beckman SW 40 cellulose nitrate centrifuge tubes. The gradient was composed of 1 ml of the solution with density of 1.3 ; 2 ml of the solution with density of 1.4 , and 3 ml of the solution with density of 1.5. Each step gradient was topped with 2 to 3 ml of the freshly polyethylene glycol precipitated bacteriophage suspension which was also layered on with the peristaltsic pump. The step gradients were centrifuged in an SW 40 rotor at 22 000 rpm ( 33 0000 x g) for 3 hours at 4°C. The lower band containing the intact bacteriophage was removed with a Pasteur pipette, placed in dialysis tubing and dialysed against a thousand fold volume of 0.1 M Tris buffer for 1 hour at 4°C. The dialysis sac was transferred to a fresh flask of buffer and dialysed against an additional thousand fold volume of buffer for 1 hour at 4°C. The resultant purified bacteriophage was stored at 4°C. If bands could not be visualized in the gradients, the gradient was fractioned into ~20 drop fractions by placing the micropipette of the peristalsic pump to the bottom of the gradient and pumping the gradient out into disposable 12 x 75 mm disposable test tubes. A sample of the fractions were diluted 1 in 10 in Lambda diluent (see section 2.3.3) and the absorbance of the diluted sample was read at 230, 260 and 280 nm wavelengths in a Gilford 250 Spectrophotometer. The fractions exhibiting a peak at 260 and 280 nm were pooled and dialysed just as the bacteriophage removed with the Pasteur pipette.

**2.10 Propagation of Bacteriophage T4 and λ:**

**2.10.1 T4** : (Winkler *et al.* 1976) A culture of *E. coli* 364 was grown in 100 ml of LB overnight to stationary phase. Thirty milliliters of the overnight culture were used to innoculate 1 liter of LB in a 4 liter Erlenmyer flask. The flask was placed on a shaker incubator at approximately 100 rpm (at a setting of 4) at 37°C. The optical density of the culture at 600 nm was monitored with a spectrophotometer every 20 minutes until it reached an absorbance reading of 0.12 (corresponding to $4 \times 10^8$ cells per ml). Bacteriophage T4 ($2 \times 10^{10}$) was added and the culture mixed immediately. The infected culture was incubated on the shaker incubator for 4 hours, approximately 5 ml of chloroform was added to the flask. The flask was allowed to sit at 37°C without shaking for 30 minutes and was then transferred to an ice bath for a further 30 minute incubation.. The bacterial debris was removed by centrifugation of the bacteriophage lysate at 8 000 x g for 30 minutes. The supernatant was stored at 4°C in glass Wheaton bottles. The titer of bacteriophage T4 in the lysate was determined.

**2.10.2** λ (modification of procedure in Manniatis *et al.* 1982 by Dr. A. A. Ahmed, Department of Genetics, University of Alberta ) : A culture of *E. coli* 176 was grown in 100 ml of LB supplemented with 0.05 M $MgSO_4$ (stock 1 M $MgSO_4$ in double distilled water) overnight on a shaker incubator at 37°C to stationary phase. To 0.5 ml of the overnight culture dispensed into sterile 13 x 100 mm screw-capped tubes, 0.1 ml of the bacteriophage λ lysate ( $2 \times 10^8$ PFU / ml) was added. For adsorption, the bacteriophage were allowed to incubate with the bacterial cells without shaking for 20 minutes at 37°C. The cell-bacteriophage mixture was added to 2.5 ml of L-sloppy agar at 46°C, mixed by inversion, and poured onto LA plates which had previously been allowed to come to room temperature. The soft agar overlay LA plate was incubated for 7 to 8 hours at 37°C. The plates were then flooded with 3 ml of Lambda

diluent and placed on a shaking stir plate at 4°C for 1 hour. The L-sloppy agar and the Lambda diluent were transferred to a Corex tube with the aid of a Pasteur pipette and a rubber policeman and centrifuged at 8 000 x g for 20 minutes. The supernatant was filter sterilized with a 0.22 µm Millipore filter and titrated. The bacteriophage lysate was stored at 4°C in sterile Wheaton bottles.

**2.11 Determining the Titer of T4 and λ :**

The same technique was used to titer both the bacteriophage λ and T4 with the exception of the following modifications : i) host strains (*E. coli* 364 was used for T4 whereas *E. coli* 176 was used with λ), ii) media (LB supplemented with 0.05 M $MgSO_4$ was used to grow *E. coli* 176 and bacteriophage λ. Unsupplemented LB was used to grow *E. coli* 364 and T4), adsorption time (bacteriophage λ was incubated with the host cells for 20 minutes at 37°C prior to adding sloppy agar whereas the bacteriophage T4 and the host cells were just mixed prior to adding the sloppy agar). A 100 µl sample of the bacteriophage lysate to be titered was serially diluted in Lambda buffer to a final dilution of $10^{-8}$. A 0.2 ml sample of a mid-log phase culture of the host bacteria was infected with 0.1 ml of the diluted bacteriophage. The cell-bacteriophage mixture was added to the L-sloppy agar at 46°C and poured onto room temperature LA plates. A control plate of uninfected host cells was always prepared. The plates were incubated at 37°C for 8 to 12 hours and the resulting plaques counted with the aid of a colony counter.

**2.12 Electron Microscopy:**

Bacteriophage L1 concentrated by polyethylene glycol precipitation was used for the electron microscopic analysis. The bacteriophage L1 was prepared for

transmission electron microscopy by negatively staining with 2% wt./vol.ammonium molybdate on formvar-coated, 200-mesh copper grids.

**2.13 Surface Labelling of Bacteriophage L1, T4, and λ:**

**Preparation of Iodo-Gen™ tubes**: Disposable (12 x 75 mm) test tubes were coated with 1 µg of Iodo-Gen™ by distributing 100 µl of a 0.01µg/µl solution (1 µl of 40 µg/µl stock in 4 ml of chloroform) into each tube. To remove the chloroform, the tubes were placed under a gentle stream of nitrogen gas or left in a fume hood until dry. The Iodo-Gen™ coated test tubes were stored at 4°C until use.

**$^{125}$Iodination of the Bacteriophage**: The bacteriophage preparations to be iodinated were purified on CsCl gradients and dialyzed against 0.1 M Tris-HCl prior to use. A 100 µl sample of the bacteriophage (with a titer of $10^{10}$ PFU/ml) was placed in the Iodo-Gen™ coated tubes followed immediately with 4 µl of stock $^{125}$I (high specific activity, New England Nuclear Corp., Boston, Mass.). The tube was allowed to incubate at room temperature for 10 minutes with intermittant swirling. One hundred microliters of a 10% trichloroacetic acid was added to the tube and the tube placed on ice for 20 minutes. The tubes were then centrifuged in the cold at 1600 x g for 30 minutes in an Eppendorf centrifuge. The supernatant fluid was decanted and the tubes left to drain in a fume hood for 30 minutes at room temperature.

**2.14 SDS-PAGE Protein Pattern :** (Laemmli 1970, Peppler 1982)
The component bacteriophage proteins were observed in $^{125}$ I surface labelled bacteriophage and unlabelled purified bacteriophage preparations. To the tubes containing the TCA precipitated $^{125}$ I surface labelled bacteriophage, a 50 µl sample of CsCl gradient purified bacteriophage was added. Fifty microliters of Laemmli

digestion mix was added to the bacteriophage preparations and the suspension neutralized to the correct pH (the suspension was changed from a yellow colour to the blue colour) using 10 N sodium hydroxide added with a micropipette. The tubes were then incubated for 5 minutes in a boiling water bath. A 5 µl sample was taken to determine the specific radioactivity of the bacteriophage samples using the gamma counter. The bacteriophage samples were adjusted such that 10 000-20 000 CPM were added per lane of a 14% SDS- PAGE mini-gel ( BIO-RAD). Low molecular weight protein standards were loaded onto the gel. The gels were run at 5 Watts constant power for approximately 1 hour, stained with Coomassie brilliant blue or silver stain, dried and exposed to X-ray film (Kodak Diagnostic Film GBX-2) at -70°C for various times ranging from 30 minutes to eight hours or alternatively left for various times or overnight at 4°C.

## 2.15 Staining of the SDS-PAGE Mini-gels

**2.15.1 Silver staining** : ( Tsai and Frasch 1982 modified by M. S. Peppler, Department of Medical Microbiology and Infectious Diseases, University of Alberta)

The gel was fixed overnight by gentle gyrotory shaking in 800 ml of 40% ethanol, 5% acetic acid solution in a covered large disposable Petri dish (Fisher 150 x 15 mm). The gel washed 3 times for 15 minutes each with 800 ml of deionized water. The water was poured off and the 148 ml of silver stain reagent was added and allowed to react with the gel with mixing for 10 minutes. The silver stain reagent was prepared just prior to use by the following procedure: 0.28 ml of 10 N NaOH was added to 27.7 ml of distilled water, followed by 2 ml of a 29% ammonium hydroxide solution, and, with vigorous mixing, 5 ml of 20% wt./vol. silver nitrate solution and finally, 115 ml of

distilled water (see 2.3.11). The silver stain reagent was poured off and the gel was washed 3 times for 10 minutes each with 800 ml deionized water. The water was poured off and 400 ml of developer (see 2.3.11) was added and the bands on the gel allowed to appear to the desired intensity or until the background turned yellow. Some of the bands on the gel appeared as white bands on a yellow background therefore the developer was allowed to turn the background very yellow for a contrast in some of the gels. This overexposed the positively stained bands so two separate gels were done. The developer was poured off after the bands or background had developed to the desired intensity and a solution of 7% acetic acid was added to stop the development. The gel was photographed and dried on a slab gel drier (BIO-RAD Dual Temperature Slab Gel Drier Model 1125 B). An autoradiograph of the gel was done (see 2.14)

**2.15.2 Coomassie Brilliant Blue**: The minigels were fixed and stained in 0.2% Coomassie brilliant blue R-250 dissolved in 7% acetic acid-5% isopropanol fixing solution for approximately 2 hours. After staining, the gels were destained by washing in 7% acetic acid-5% isopropanol mixture until the background was light coloured. The gel was washed in a final destaining solution of 7% acetic acid. The gel was photographed and dried on the slab gel drier (BIO-RAD Dual Temperature Slab Gel Drier Model 1125 B). An autoradiograph of the gel was done (see 2.14)

**2.16 Nucleic Acid Determination:** (Burton 1956, Paranchych and Graham 1962)

Bacteriophage L1 and T4 samples were prepared for analysis by concentration of crude bacteriophage lysates by polyethylene glycol precipitation, purification by separation on a cesium chloride step gradient followed by dialysis of the sample against 0.1 M Tris buffer. Samples of the bacteriophage ( 0.05-1 ml volumes) were placed

into test tubes. Calf thymus DNA (from a stock solution of 1.25 mg/ml in distilled water) was added to another set of test tubes such that a standard curve ranging from 12.5 to 125 µg of DNA was produced. One milliliter of 0.5 N perchloric acid was added to both the tubes containing bacteriophage and calf thymus DNA and heated at 80°C for 15 minutes. A tube of 1 ml of perchloric acid was also made as a blank for the following diphenylamine spectrophotometric assay. The blank was also heated to 80°C for 15 minutes. The tubes were all allowed to cool to room temperature. Two volumes of diphenylanime reagent containing acetaldehyde (see section 2.3.32) was added to each tube and mixed by inversion. The tubes were placed in a 30°C water bath and the colour allowed to develop for 16-20 hours. The absorbance at 600 nm of each tube was measured against the blank and compared with the values obtained with the standard DNA wavelength using the spectrophotometer (Coleman Junior® II Spectrophotometer).

**2.17 Bacteriophage DNA Isolation :** (Maniatis *et al.* 1982)

The bacteriophage that had been purified on the CsCl gradients and had produced a thick, visible band in the gradient was dialyzed against 0.1 M Tris (pH 8) and placed in an Eppendorf tube such that it was only one third full (400 µl of purified bacteriophage L1). EDTA from a stock solution (0.5 M, pH 8.0) was added to each tube to give a final concentration of 20 mM (approximately 20 µl stock EDTA). Proteinase K from a stock solution (1mg/ml) was added to a final concentration of 50 µg/ml (approximately 20 µl stock Proteinase K). SDS from a stock solution (20% wt./vol in water) was added to a final concentration of 0.5% (approximately 11 µl stock SDS). The tube was inverted several times to mix. The tubes were placed in a 65°C water bath and incubated for 1 hour. An equal volume of equilibrated phenol was

added to each tube. The tubes were mixed by inversion and the phases separated by centrifugation at 1600 x g for 5 minutes at room temperature. A wide bore pipette was used to transfer the aqueous phase to a clean tube. The aqueous phase was extracted once with a 50:50 mixture of equilibrated phenol and isoamyl alcohol-chloroform. The phases were separated by centrifugation at 1600 x g for 5 minutes at room temperature. The aqueous phase was transferred to a clean tube and extracted once with an equal volume of isoamyl alcohol-chloroform. The aqueous phase was recovered and extracted with an equal volume of diethyl ether. The phases were allowed to separate by leaving the tube in a vertical position undisturbed for 10 minutes at room temperature. The ether phase was removed with a Pasteur pipette, re-extracted with an equal volume of diethyl-ether, and the phases allowed to separate. The ether was removed with a Pasteur pipette and residual ether removed by blowing a stream of nitrogen gas across the surface of the aqueous phase in a fume hood. A 0.1 x volume of 3 M sodium acetate and 2.5 x volume of 95% ethanol was added to the tubes to precipitate the DNA. The DNA solutions were stored at -20°C overnight. To recover the DNA, the precipitated DNA was centrifuged at 1600 x g for 15 minutes in the cold. The supernatant was decanted and the DNA pellet washed in 3x volume of ice-cold 70% ethanol. The DNA pellet was dried under vacuum and resuspended in 50 µl of distilled water or TE buffer. The DNA solution was heated to 65°C for 30 minutes to aid the DNA to enter solution and then stored at 4°C for at least 2 days before use to ensure the DNA was fully dissolved.

**2.18 Restriction Enzyme Analysis of Bacteriophage DNA :**

**2.18.1 Restriction Enzyme Profile** : Several restriction enzymes were used under their specific conditions to digest samples of the bacteriophage L1 DNA. A control sample of DNA (λ or suitable plasmid) was also digested in order to ensure the restriction enzyme was active. The restiction enzyme digests were run on 1% Tris borate (TB) agarose gels or 0.6% Tris-acetate (TA) agarose gels using the Wide-mini-sub™ cell electrophoresis apparatus (BIO-RAD) and stained with 1% ethidium bromide solution. To separate the large fragments of the *Xba* I digests, a larger gel apparatus was used (gel bed 210 x 130 mm produced by Aqueboque Machine Shop, Box 205 Main Road, Aqueboque, New York 11931).

**2.18.2 Restriction Enzyme Map** : The restriction enzymes that produced 5 or less bands on the gels were used for restriction mapping. Double digests using two enzymes were conducted. The restriction enzyme digestion requiring a low salt buffer was conducted first if the two enzymes required different reaction conditions. If a common buffer was used, the two enzymes were added at the same time. The resulting digestions were run on 1% TB agarose gels and 0.6% TA agarose gels. To look for small fragments (<1 kb), 2% agarose and 14% PAGE gels were run.

**2.18.3 Analysis of the Large *Xba* I Fragment** : In order to determine which of the *Sst* I / *Xba* I fragments were contained in the large *Xba* I fragment, a sample of L1 DNA was digested with *Xba* I, separated on a 0.7% TB agarose gel, stained with ethidium bromide and the large band removed from the gel with the aid of a razor and a UV-lightbox. The DNA was isolated from the gel using Gene-Clean™. The re-isolated DNA fragment was digested with *Sst* I and separated on a 0.7% TB agarose gel. The resulting gel was stained with ethidium bromide and photographed.

## 2.19 Analysis of the Bacteriophage L1 DNA Ends

### 2.19.1 End-Labelling : (Maniatis *et al.* 1982)

Bacteriophage L1 DNA (7-8 µg) was placed in an Eppendorf tube and dried down in a vacuum dessicator. To the dry DNA the following were added : 2 µl of 10 x kinase buffer, 10 µl $^{32}$P-γ-ATP, and 8 µl of water. To the DNA solution, 1 µl of T4 polynucleotide kinase was added and the tube incubated at 37°C for 30 minutes. To stop the reaction, 5 µl of 0.25 M EDTA was added and the tube vortexed. The DNA solution was then phenol extracted with an equal volume of equilibrated phenol and ethanol precipitated with 0.1 x volume of 3 M sodium acetate and 2 x volume of 95% ethanol and incubated on dry ice for 30 minutes. The precipitated DNA was pelleted by centrifuging in the cold for 10 minutes. The supernatant was decanted and the DNA pellet dried in a vacuum dessicator. The DNA was resuspended in 20 µl of water. Two 2 µl samples of the end-labelled DNA were digested with restriction enzymes (*Xba* I and *Sst* I) known to give few bands on digestion. The restriction enzyme digested DNA was run on a 1% TB agarose gel. The gel was then dried on a slab gel drier for 2 hours, exposed to X-ray film and the autoradiograph developed. The actual goal of the experiment was to end-label the bacteriophage L1 genome, subject it to restriction enzyme digestion with the enzymes being used for mapping (*Xba* I and *Sst* I) , run a 1% agarose TB gel and expose the gel to autoradiography as a means of determining the restriction fragments that were at the ends of the bacteriophage L1 DNA.

### 2.19.2 DNA Reassociation Experiment : (Dr. A. R. Morgan, Department of Biochemistry, University of Alberta )

The following experiment was done to determine if there were any crosslinking or hairpin loops at the ends of the DNA of the bacteriophage L1. A sample of

bacteriophage L1 DNA (1 µl of a stock DNA solution of 1 mg DNA/ml of water) was placed in a test tube. A sample of calf thymus DNA (from a stock of 1 mg/ml) was prepared in the same manner to act as a control. A 5.0 ml volume of ethidium bromide buffer pH 8.0 (see 2.3.7.) was added to the tubes and the tubes allowed to sit at room temperature for 1 minute. The absorbances of the tubes were read in the fluorometer. The tubes were then transferred to a water bath at 96°C and allowed to incubate for exactly 2 minutes. The tubes were then transferred to a room temperature water bath for exactly 2 minutes and the absorbance of the DNA solutions taken and compared.

**2.20 Nick-Translation:** (Maniatis *et al.* 1982)

Two hundred nanograms of bacteriophage L1 DNA was added to an Eppendorf tube followed by 2 µl of three cold nucleotide mixtures ( stock solutions were 1 mM dNTP in 5 mM Tris-HCl pH 7.5, 2 mM ß-mercaptoethanol, 1 mM EDTA, 50% ethanol and stored at -20°C). The nucleotide mixtures used were dTTP, dCTP, and dGTP. The DNA and nucleotide solutions were dried down under vacuum. The following were added to the dry tube: 160 µl of water, 2 µl of 10x nick translation buffer (50 µl of 1 M Tris-HCl pH 7.8 0.1 M $MgCl_2$ , 1 µl of 2 mg/40 µl BSA, 49 µl water, 0.7 µl ß-mercaptoethanol. This buffer is made up just prior to use and stored on ice.), 2 µl 10x DNase (from a stock of 1 mg/ml in water and diluted to $10^{-7}$) and 2 µl of $\alpha^{32}$P- dATP (New England Nuclear specific activity 3 000 Ci/mMole). The tube was incubated at 12°C for 30 minutes. DNA polymerase I (0.25 µl ) was added to the tube and allowed to incubate for a further 1.5 hours at 12°C. Eight microliters of 0.25 M EDTA was added to the tube and the tube was incubated at 65°C for 10 minutes. The contents of the tube were passed through a G75 column in TE buffer to separate the unincorporated

label from the labeled DNA. A sample of the labelled DNA was counted in the scintillation counter (Beckman LS 9800) to determine the specific activity.

## 2.21 Isolation of Bacterial DNA :

**2.21.1 Mini-Method:** (Maniatis *et al.* 1982 modified by B. Pasloske in the laboratory of Dr. W. Paranchych, Department of Biochemistry, University of Alberta.) The organisms were grown in broth culture to stationary phase. An Eppendorf centrifuge tube was filled with the bacterial culture and the bacteria pelleted by centrifugation for 5 minutes at 12 000 x g in a benchtop centrifuge (Eppendorf 5413) The supernatant was decanted. The bacterial cells were resuspended in 100 µl of solution 1 (0.025 ml 1 M Tris-HCl pH 8, 0.005 ml 40% glucose, 0.95 ml distilled water, 0.04 ml 0.25 M EDTA pH 8 and a small spatula amount of lysozyme and 10 µl of 5 mg/ml RNase added just prior to use) by vortexing. The tubes were allowed to stand at room temperature for 10 minutes. Two hundred microliters of solution 2 (0.050 ml 10% SDS and 0.95 ml distilled water) was added to each tube, the tubes vortexed to mix and allowed to sit at room temperature for 10 minutes. Seven hundred microliters of TE buffer was added to each tube. To break up the pellet and facillitate the extraction of the DNA, the contents of each tube was pulled through a syringe (16 gauge bore) several times. The bacteria were phenol extracted twice with 400 µl of equilibrated phenol. Each preparation was split into two tubes containing 450 µl each. Fifty microliters of 3 M sodium acetate was added and the tubes mixed by vortexing. The tubes were then filled with cold 95% ethanol and the DNA precipitated by storing at -20°C overnight or -70°C for 15-20 minutes. The DNA was spun down by centrifuging for 5 minutes in an Eppendorf centrifuge in the cold. The DNA was dried in a vacuum dessicator and resuspended in 50 µl of TE buffer.

**2.21.2 Large-Scale Chromosomal Isolation** : (Maniatis *et al.* 1982 modified by Dr. W. Paranchych laboratory, Department of Biochemistry, University of Alberta).

A broth culture of the bacteria was grown into stationary phase and a 40 ml aliquot placed in a Corex tube and centrifuged at 8 000 x g for 20 minutes to pellet the cells. The cells were resuspended in 2 ml of 25 mM Tris-HCl (pH 8.0) and 6 ml 10 mM Tris-HCl / 1 mM EDTA / 20% sucrose (wt /vol) pH 8.0 and left on ice for 10 minutes. Four milligrams of lysozyme were added to each tube. The tubes were vortexed and left on ice for 10 minutes. One hundred sixty micrograms of 10% SDS were then added to the tubes. The tubes were vortexed and kept on ice for 10 minutes. The tubes were then incubated at room temperature for 5 minutes and then at 70°C for 5 minutes. Eight milliliters of TE buffer was added to each tube and the lysate passed several times through a 10 ml syringe with a 16 gauge needle to mix the solution and shear the DNA. The lysate was phenol extracted three times with 5 ml volumes of equilibrated phenol. The phases were separated by centrifugation at 8 000 x g for 10 minutes. Boiled RNase was added to a final concentration of 100 µg/ml and left at room temperature for 1 hour. Phenol extractions were continued until the protein interface disappeared. A 0.1 x volume of 3 M sodium acetate and 2x to 3x volume of 95% ethanol were added to the tubes. The tubes were then inverted several times to clump the DNA which was then removed using a glass hook made from a Pasteur pipette. The DNA was dissolved in 7 ml of TE buffer and 1.05 ml of 3 M sodium acetate was added. The DNA solution was phenol extracted once more and then 18 ml of 95% ethanol was added to precipitate the DNA. The tubes were stored overnight at -20°C and then centrifuged at 10 800 for 20 minutes. The DNA pellet was resuspended in 1 to 2 ml of TE buffer.

## 2.22 Preparation of Nitrocellulose Filters for Bacteriophage L1 DNA Hybridization :

### 2.22.1 Colony Hybridization: ( Maniatis *et al.* 1982)

This method was used to screen selected strains of three *Bordetella* species; *B. pertussis* , *B. parapertussis* , and *B. bronchiseptica*. Pieces of nitrocellulose (BIO-RAD Trans-Blot Transfer Medium 0.45 µm) were cut so as to fit on the agar surface of the BGA plates. A grid was drawn onto ordinary loose-leaf paper that was the same size as the nitrocellulose, dividing up the surface into regular pieces. With the nitrocellulose still within the protective covering paper, the grid was traced onto the nitrocellulose by using a pen to press the impression of the grid onto the underlying nitrocellulose. The nitrocellulose filters were placed in plastic petrie dished and gas sterilized (Anprolene™). Just prior to use, nitrocellulose filters were placed onto the BGA agar surface using sterile forceps. The bacterial strains to be screened were grown up on BGA plates at 37°C in a humidified incubator. To compensate for the different growth rates of the three species, 84 hour *B. pertussis*, 36 hour *B. parapertussis* and 18 hour *B. bronchiseptica* cultures were used to inoculate the nitrocellulose filter. The inoculation of the filter was spread over three days. The first day, all *B. pertussis* strains were inoculated, the second day, all *B. parapertussis* strains were inoculated, and on the third day all *B. bronchiseptica* were inoculated. A negative control, *Escherichia coli* 364 was also inoculated at the same time as the *B. bronchiseptica*. Inoculation was done by random sampling of the bacterial colonies on the BGA plates with a sterile toothpick and transferring the growth to a grid on the nitrocellulose-BGA plates. The nitrocellulose-BGA plates were incubated at 37°C in a humidified incubator for a total of four days. The nitrocellulose was removed from the

BGA plate and the colonies lysed on the nitrocellulose by the following method : the nitrocellulose was placed bacteria side-up (avoiding air bubbles) for 3 minutes onto a Whatman 3 MM filter that had been soaked with a 10% SDS solution. Excess SDS was removed by scraping the bottom of the filter against the side of the tray containing the SDS-soaked filter. The nitrocellulose was then transferred to a tray containing 0.5 M NaOH and 1.5 M NaCl and incubated for 5 minutes. Excess liquid was removed against the side of the tray and the nitrocellulose transferred to a tray containing 0.5 M Tris-HCl (pH 8.0) and 1.5 M NaCl and incubated for 5 minutes. Excess liquid was removed and the nitrocellulose placed in a tray containing 2 x SSPE and incubated for 5 minutes. The nitrocellulose was placed onto absorbant filter paper and a paper towel and baked at 65°C for a minimum of 2 hours in an oven. The nitrocellulose was prepared for bacteriophage L1 DNA hybridization.

**2.2.2 Dot Blot Hybridization:** ( Maniatis *et al.* 1982)

This method was used to screen those colonies which showed some degree of hybridization with the bacteriophage L1 DNA probe but less than the positive controls. The bacterial DNA used was extracted by the mini-method of bacterial DNA extraction. The concentrations of the resulting DNA solutions were estimated by adding a sample of the DNA solution to 5.0 ml of the ethidium bromide buffer (see section 2.3.7) and measuring the fluoresence (Gilson Spectro/glo Filter Fluorometer). The volume of bacterial DNA solutions placed on gridded nitrocellulose was calculated so as to place a standard amount of DNA on each grid. After application of the DNA, the nitrocellulose was baked at 65°C for a minimum of 2 hours in an oven.

**2.2.3 Southern Blot Hybridization:** (Maniatis *et al.* 1982)

This method was used to determine if the colonies shown to be positive by the dot blot had the bacteriophage L1 genome incorporated into the bacterial genome in

order to determine if the bacteriophage was lysogenic. The bacterial DNA used was prepared by the large-scale DNA extraction. The bacterial DNA was digested by the restriction enzyme *Xba* I according to manufacturers instruction and the resulting digested bacterial DNA run on a 0.75% agarose TB gel. The gel was stained with ethidium bromide and photographed. The DNA fragments were transferred from the agarose gel to nitrocellulose paper using the following steps. The gel was transferred to a glass dish and unused areas of the gel were trimmed away using a razor blade. The DNA was denatured by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH for 20 minutes with constant shaking at room temperature. The gel was neutralized by transferring the gel to a tray containing several volumes of a solution of 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl and soaking the gel for 20 minutes at room temperature with constant shaking. A piece of nitrocellulose paper was cut such that it fit the gel surface without overhang. Two pieces of Whatman 3 MM paper were cut, one was cut slightly larger than the gel and the other cut slightly smaller than the gel. A small tray was completely filled with a sponge (regular kitchen type). The sponge was moistened with a 10 x SSPE solution but there was no liquid above the surface of the sponge. The larger Whatman 3 MM paper was placed on the soaked sponge followed by the gel with the DNA side up (same side up that was up during the electrophoresis) followed by the cut nitrocellulose paper followed by the smaller Whatman 3 MM paper followed by a stack a paper towels cut to the same size as the smaller Whatman 3 MM paper and a small weight. Care was taken to ensure that the liquid in the sponge could get to the paper towels only through the gel and not through any material overhanging the gel which would prevent the transfer of the DNA. The system was left undisturbed at room temperature overnight. The nitrocellulose was removed from the gel and baked at 65°C for a minimum of 2 hours.

**2.23 Hybridization of Nick-Translated Bacteriophage L1 DNA to Bacterial DNA:**

( Maniatis *et al.* 1982 modified by W.Paranchych laboratory, Department of Biochemistry, University of Alberta)

The colony blots, dot blots and Southern blots were used in the hybridization experiments. The blots were preincubated in the following solution: 2.5 ml of 20 x SSC, 0.5 ml of 2% SDS, 0.040 ml of 0.25 M EDTA, 1 ml of 10 x PM (0.06 g Ficoll, 0.06g BSA, 0.06g Polyvinyl pyrrolidone, 15 ml water, 15 ml 20 x SSC), 0.98 ml distilled water, and 5 ml of formamide. The blots were placed in a heat-sealable plastic bag (seal-o-bag), sealed, and incubated with constant shaking at 37°C for at least 3 hours. The pre-hybridization mix was poured off. A mixture of denatured probe and calf thymus DNA was prepared by boiling 1 to 3 x $10^6$ CPMs of nick-translated probe and 0.1 ml of calf thymus DNA (from a stock of 5 mg/ml which had been sheared in a sonicator) for 3 minutes. The denatured probe mix was added along with 2 to 4 ml of the pre-hybridization buffer to the plastic bag with the blots. The bag was resealed and incubated at 37°C overnight with constant shaking. The hybridization mix was poured off and the blot removed from the plastic bag and placed in a glass tray. The blot was rinsed with the following solution at 65°C for 30 minutes with constant shaking : 125 ml of 20 x SSC, 5 ml 10% SDS, 2 ml 0.25 M EDTA, and 368 ml water. The blot was then washed twice at room temperature with 2 x SSC, air dried and exposed to X-ray film ( Kodak Diagnostic Film GBX-2) overnight.

# 3.0 RESULTS

## 3.1 Isolation of Bacteriophage L1.

### 3.1.1 Titer of Bacteriophage L1 on Broth Culture Isolation.

Bacteriophage L1 was obtained from *B. bronchiseptica* AG1-L broth culture (see section 2.4) after 5 to 6 hours of incubation at 37°C (titer ~ $10^5$ PFU/ml).

### 3.1.2. Plaques on Indicator Organism:

Bacteriophage L1 formed clear plaques measuring 1 to 2 mm on the indicator organism, *B. parapertussis* 17903 after 24 hours of incubation. Indicator plates made using TSA plates more than 2 weeks old produced plaques pinprick in size. Conversely, use of TSA plates less than 24 hours old produced smeared plaques of 2 to 3 mm in diameter after 24 hours of incubation. Standard plaque size was obtained on indicator plates made from TSA plates that were 2 to 10 days old.

## 3.2 Soft-Agar Overlay Method of Bacteriophage Propagation.

The soft-agar overlay method was used to raise the titer of the bacteriophage L1 lysates obtained from plaque-purified bacteriophage L1 to ~$10^9$ PFU/ml. The soft agar overlay method proved to be more labour intensive and time consuming than did the broth method (see section 3.4) and was therefore not used for mass production the bacteriophage L1 necessary for characterization.

### 3.2.1 Effect of Ambient Temperature on Lysis of the Soft-Agar Overlay Plates

After pouring the soft-agar overlays, the overlays were allowed to congeal (without stacking) on the cold metal of the biological hood. The plates were then stacked and incubated in the cold room (~ 4°C) for at least 30 minutes prior to

incubation at 37°C. Incubation of the overlays at 4°C prior to incubation at 37°C was instituted after an apparent effect of temperature on the plaquing ability of the bacteriophage L1 was observed. Numerous soft-agar overlays were poured and stacked 10 high in the biological hood to save space. After incubation (at 37°C without the 4°C incubation), the plates exhibited a gradient of lysis from the top of the stack to the bottom ranging from less than 10% lysis for the uppermost plate to 95% lysis for the bottom plate. This was a reproducible occurance especially when the temperature in the hood was elevated above room temperature by using the Bunsen burner flame in the hood.

### 3.2.2 Optimal Harvest of the Soft-Agar Overlay Lysates.

Parameters for harvesting the bacteriophage L1 that were found to be important to the titer of the bacteriophage lysates are as follows : plates that exhibited 90% to 95% lysis produced maximal harvests; plates left in the incubator at 37°C for longer than ~30 hours produced bacteriophage lysates of lower titers; and higher titers of bacteriophage were harvested if the Lambda diluent was removed without the soft-agar from the soft-agar overlay plates. To determine if harvested lysates would be of higher titer if the soft-agar was removed from the plates along with the Lambda diluent (see section 2.8.1), the following simple experiment was conducted. Two sets of 10 soft-agar overlay plates, infected and incubated under the same conditions, were flooded with 3.0 ml of Lambda diluent. To harvest the bacteriophage, one set of plates had the soft-agar scraped off with a rubber policeman and harvested with the Lambda diluent and the other set was harvested with only the Lambda diluent. The titers of the resulting bacteriophage lysates were $2 \times 10^8$ PFU/ml for the lysates with the soft agar and $1 \times 10^9$ PFU/ml for the lysates without the soft agar. The lower titer of the lysate harvested

with the soft agar as compared with the lysate harvested without the soft agar contraindicated the harvest of the soft-agar with the Lambda diluent. As a further attempt to harvest more bacteriophage from the soft-agar overlays, a second wash of 3 ml of Lambda diluent was added to the soft-agar overlay plates after the first wash had been harvested. The plates with the second wash were incubated at 4°C with gentle rotary shaking for a further 2 hours. The titers of the two lysates were as follows : 1.7 x $10^9$ PFU/ml for the first wash and 5 x $10^7$ PFU/ml for the second wash. The titer of the lysate from the second wash did not warrant its inclusion into the protocol.

### 3.2.3 Parameters Influencing the Titer of the Bacteriophage Lysates.

Once the optimal harvesting technique was established (see section 3.3.2) attempts were then made to maximize the titers of the bacteriophage lysates produced by the soft-agar overlay method by manipulating the parameters listed below (sections 3.2.3.1 to 3.2.3.3).

#### 3.2.3.1 Bacteriophage Adsorption at Room Temperature.

Host bacterial cells were infected as described in the soft-agar overlay method of bacteriophage propagation (see section 2.8.1) however, with a modification to the protocol which allowed the bacteriophage L1 to adsorb to the host cells by pre-incubation of the bacteriophage L1 with the host cells at room temperature prior to being added to the soft agar. Room temperature pre-incubation for the adsorption of the bacteriophage l to its host had been successfully used to obtain maximal titers (A. A. Ahmed, Department of Genetics, University of Alberta personal communication). The resultant titer of bacteriophage L1 lysates however, showed no significant difference in the titers of the lysates produced after the 15 minute pre-incubation at

room temperature as compared to the lysates produced using the protocol without the room temperature pre-incubation. The average of the titer of the bacteriophage lysate without pre-incubation was $1.95 \times 10^9$ PFU/ml $\pm$ $6.4 \times 10^8$ PFU/ml. The average titer of the bacteriophage lysate with a 15 minute pre-incubation at room temperature was $1.90 \times 10^9$ PFU/ml $\pm$ $9.2 \times 10^8$ PFU/ml.

**3.2.3.2 The Effect of Calcium and Magnesium Ions on the Titers of Bacteriophage L1:**

Certain bacteriophage-host cell systems require calcium or magnesium ions for maximal bacteriophage adsorption (Manniatis *et al.* 1982) and cation requirement is tested in characterization of bacteriophage using the criteria outlined by the I.C.T.V. (Ackermann 1987). Bacteriophage L1 was propagated by the soft-agar overlay method (see section 2.8.1) using media supplemented with 10, 20, 50, 75, 100 mM of $CaCl_2$ or $MgCl_2$. The host cells were infected at a multiplicity of infection of 0.02. A control plate of unsupplemented infected host cells was also plated. The resultant titers of the bacteriophage L1 lysates were in the range of $1 \times 10^9$ to $5 \times 10^9$ PFU/ml which was comparable to the titer of the bacteriophage L1 lysate ($5 \times 10^9$ PFU/ml) obtained from the unsupplemented TSB and soft-agar. The bacterial lawns produced on the agars supplemented with the various concentrations of $CaCl_2$ and $MgCl_2$ were on visual inspection, exhibiting the same amount of growth as the unsupplemented lawn.

**3.2.3.3 The optimal multiplicity of infection (moi).**

Determination of the optimal multiplicity of infection is crucial to produce maximum titers of bacteriophage in the lysates (Adams 1959). The host cells, *B. parapertussis* 17903 were infected as described in the soft-agar overlay method of

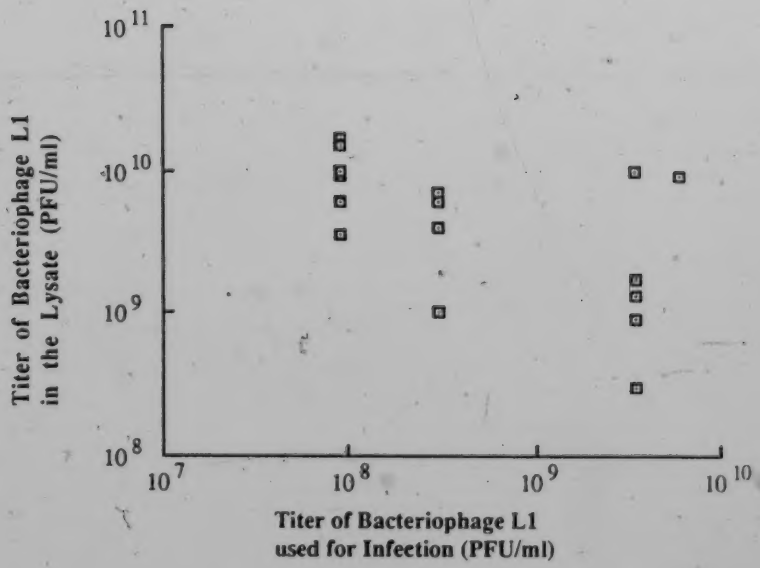
bacteriophage propagation (see section 2.8.1). A rough estimate of the optimal multiplicity of infection was determined from various infections used in several soft-agar overlay propagations of the bacteriophage L1. To pinpoint the value for the optimal multiplicity of infection, an additional experiment was conducted.

The initial infections using the soft-agar overlay method were conducted using several different multiplicities of infection. The original premise guiding these first experiments was that by increasing both the titer of bacteriophage used for infection and increasing the number of bacterial hosts available for infection, an increase in the resulting titer of the bacteriophage lysate should result. The first experiments used an inoculum of bacteria of 0.4 ml of *B. parapertussis* 17903 adjusted to an optical density at 540 nm of 0.8 (viable bacterial counts not done). The soft-agar overlays were infected with 50 µl of bacteriophage suspension with titers $10^6$ PFU/ml in one experiment and $10^8$ PFU/ml in another case. The titers obtained in the bacteriophage lysates were both approximately $10^4$ PFU/ml. Adams (1954) reported that interference with the growth of host cells would substatially reduce the burst size and consequently the titer of the bacteriophage lysates. Further, the build up of toxic metabolic by-products and depletion of nutrients in the growth medium would also severely impair the grow of the bacterial cells. The heavy bacterial inoculum in the soft-agar could produce conditions because of the numerous bacterial cells competing for nutrients and producing the metabolic by-products, that were not favourable for the production of large numbers of bacteriophage.

Consequently, a more conservative approach was taken to produce the bacterial lawn to be infected with the bacteriophage L1. The plaque assay (section 2.5) used a bacterial inoculum of 0.4 ml of a bacterial suspension adjusted to an absorbance at 540 nm of 0.2 to produce satisfactory bacterial lawns. The bacterial lawn inoculated with

the various titers of bacteriophage L1 for the next set of experiments was the standard one used for the plaque assays. The scatter plot of the titer of infecting bacteriophage versus titer of the resulting bacteriophage in the lysate (Figure 1) indicated that infection with a lower titer of bacteriophage in the standard bacterial lawn produced lysates with higher titers. In addition, it became apparent that infecting the standard inoculum of host cells with a standard titer of bacteriophage L1 would produce only a narrow range in titer of bacteriophage in the lysate. The most logical way to further increase the titers of the bacteriophage L1 lysates was to use a lower multiplicity of infection. This ratio could be changed either by increasing the bacteria seeded into the lawn for infection within the confines set by the earlier experiments or by lowering the titer of the infecting bacteriophage lysate or perhaps a combination of the two. Consequently, the following experiment was conducted to provide a greater insight into the soft-agar overlay propagation technique. The experiment had the following modifications made to the protocol for the soft-agar overlay propagation method (see section 2.8.1): the bacterial suspensions were adjusted to the following optical densities (at 540 nm) : 0.10, 0.16, 0.22, 0.27, 0.34 and 0.54 which corresponded to viable counts of $3.7 \times 10^8$, $5.6 \times 10^8$, $1.0 \times 10^9$, $1.1 \times 10^9$, $2.1 \times 10^9$, and $2.7 \times 10^9$ CFU/ml respectively. A stock lysate of bacteriophage L1 with a titer was $5 \times 10^9$ PFU/ml was serially diluted one in ten in TSB to produce bacteriophage suspensions with titers of $10^8$, $10^7$, $10^6$ and a final dilution of $10^5$ PFU/ml. A 0.4 ml sample of each different adjusted bacterial suspension was infected with 100 µl of each dilution of bacteriophage in a sterile 13 x 100 mm screw-capped tube. The range in multiplicitiy of infection was 3.4 to 0.0005. The infected cells were allowed to incubate for 20 minutes at 4°C and then plated by the soft-agar method of bacteriophage propagation. The titers of the resulting bacteriophage L1 lysates were determined by the plaque assay (see section 2.5). The

**Figure 1** : Titers of bacteriophage L1 lysates harvested from soft-agar overlays using various titers of bacteriophage to infect a standard bacterial suspension. The lysates were produced by infection of the standard bacterial inoculum (0.4 ml of a bacterial suspension *B. parapertussis* 17903 adjusted to an optical density at 540 nm of ~0.2) with several different titers of bacteriophage L1. After infection, the soft-agar overlays were incubated at 4°C for 20 minutes prior to transfer to the 37°C humidified incubator for 24 hours. The overlays were flooded with Lambda diluent and the bacteriophage harvested by recovery of the liquid component of the plates. This scatter plot suggested use of lower titers of bacteriophage L1 to infect the bacterial lawns increased the resulting bacteriophage L1 lysate within the limits of the compiled data.


Titer of Bacteriophage L1 in the Lysate (PFU/ml)
10 11
10 10
10 9
10 8
10 7
10 8
10 9
10 10
Titer of Bacteriophage L1 used for Infection (PFU/ml)

following trends could be discerned from the graph of the resulting titers of the bacteriophage lysates plotted against the increasing viable counts of host bacteria inoculated into the soft-agar overlays (Figure 2). The data indicated that the bacteriophage lysate titer increased as the viable counts of host bacteria available for infection increased up to a point after which, the number of viable organisms in the lawn did not appreciably increase the titer of the lysate (apparent saturation). This trend was most apparent when low titers of bacteriophage were used to infect the lawns. In addition, in four of the five test cases, the titer of the bacteriophage lysate decreased slightly when excess bacteria were added. The data also showed that higher bacteriophage lysate titers were produced when a low titer of bacteriophage was used to infect the host bacteria. Upon extrapolation of the graph (Figure 2), the data suggested that titers of $10^3$ PFU/ml or less may produce even greater yields of bacteriophage. In previous experiments with the soft-agar overlays, it was found that bacteriophage lysates with a titer of approximately $10^3$ PFU/ml did not produce lawns with 90 to 95% lysis and for this reason were excluded from the experiment (see section 3.3.2). A plot of increasing titer of infecting bacteriophage in a standard bacterial inoculum against titer of the bacteriophage lysate produced showed that increased titer of infecting bacteriophage decreased the resulting titer of the lysate (Figure 3).

**3.2.4 SUMMARY**: Therefore in general, to produce bacteriophage L1 lysates of high titer, a low multiplicity of infection should be used. The optimal multiplicity of infection was 0.0009. To improve the method listed in section 2.8.1, it would be advisable to inoculate a bacterial suspension with an optical density of ~0.3 at 540 nm with a bacteriophage lysate with a titer of ~ $10^4$ PFU / ml. Further experimentation is necessary to test this hypothesis. Conditions for the production of bacteriophage L1

**Figure 2.** Determination of the Optimal Multiplicity of Infection for the Soft-Agar Overlay Method of Bacteriophage L1 Propagation : Effect of Using Various Bacterial Suspensions for Infection with Increasing Titers of Infecting Bacteriophage L1 and the Titer of the Resulting Lysates. Each point on the graph represents the titer of a bacteriophage L1 lysate produced from infecting six bacterial suspensions of *B. parapertussis* 17903 ($3.7 \times 10^8$, $5.6 \times 10^8$, $1.0 \times 10^9$, $1.1 \times 10^9$, $2.1 \times 10^9$ and $2.7 \times 10^9$ CFU/ml) with five different titers of bacteriophage L1 ($10^5$, $10^6$, $10^7$, $10^8$, $10^9$ PFU/ml). The standard protocol for the soft-agar overlay method of bacteriophage L1 propagation was followed. The lines connecting the points reveal that the titer of bacteriophage L1 lysates reached an optimal level when the bacterial inoculum was between $1 \times 10^9$ and $2 \times 10^9$ CFU/ml, regardless of the titer of the infecting bacteriophage. In addition, the highest titer bacteriophage L1 lysate was achieved using an infecting titer of bacteriophage L1 of $10^5$ PFU/ml (50 µl) and a bacterial inoculum of $1 \times 10^9$ to $2 \times 10^9$ CFU/ml (400µl) indicating the optimal multiplicity of infection in this experiment was 0.0009.

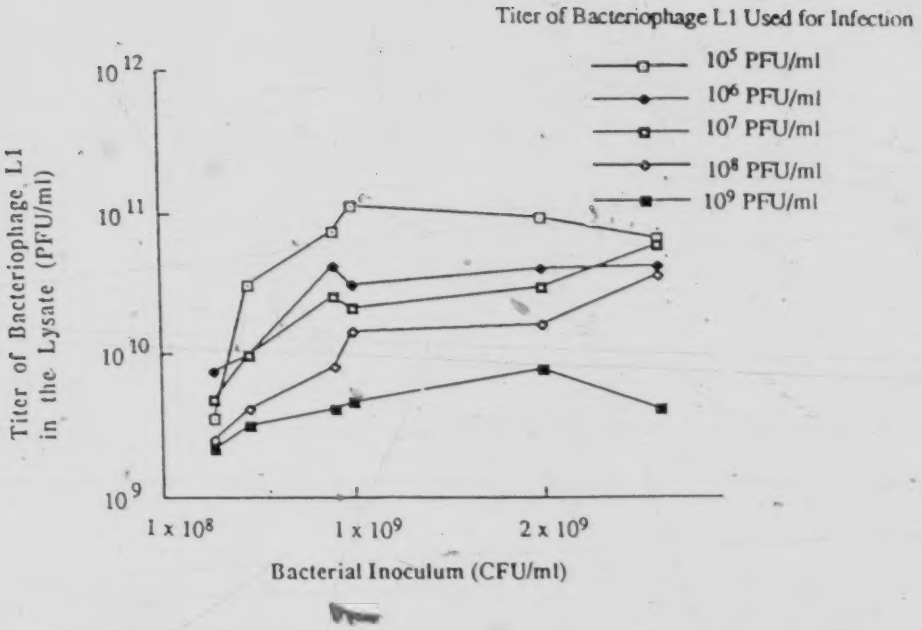
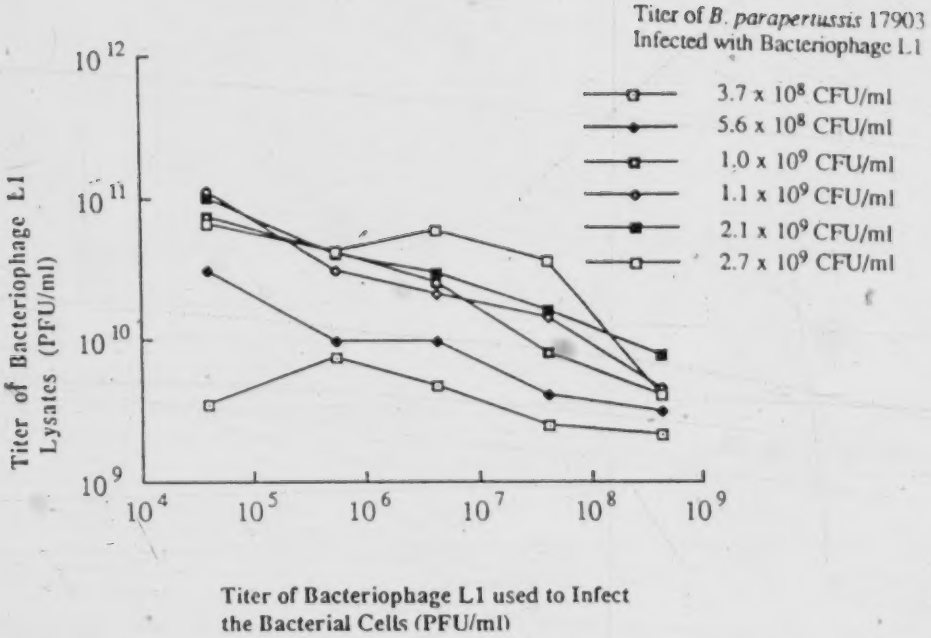

Titer of Bacteriophage L1 Used for Infection
10^5 PFU/ml
10^6 PFU/ml
10^7 PFU/ml
10^8 PFU/ml
10^9 PFU/ml
Titer of Bacteriophage L1 in the Lysate (PFU/ml)
10^12
10^11
10^10
10^9
1 x 10^8
1 x 10^9
2 x 10^9
Bacterial Inoculum (CFU/ml)

**Figure 3**. Determination of the Optimal Multiplicity of Infection for the Soft-Agar Method of Bacteriophage L1 Propagation : Effect of Increasing Titer of Bacteriophage L1 used to Infect Various Bacterial Suspensions and the Resulting Titers of the Bacteriophage L1 Lysates. Each point on the graph represents the titer of a bacteriophage L1 lysate produced from infecting six bacterial suspensions of *B. parapertussis* 17903 ($3.7 \times 10^8$, $5.6 \times 10^8$, $1.0 \times 10^9$, $1.1 \times 10^9$, $2.1 \times 10^9$ and $2.7 \times 10^9$ CFU/ml) with five different titers of bacteriophage L1 ($10^5$, $10^6$, $10^7$, $10^8$, $10^9$ PFU/ml). The standard protocol for the soft-agar overlay method of bacteriophage L1 propagation was follwed. The lines connecting the points revealed that infection of the bacterial suspensions with increasing titers of bacteriophage L1 produced bacteriophage L1 lysates of decreasing titers, regardless of the bacterial suspension being infected. The highest titer of bacteriophage L1 in the lysates was obtained using a bacterial inoculum of between $1 \times 10^9$ and $2 \times 10^9$ CFU/ml (400µl) infected with bacteriophage L1 with a titer of $10^5$ PFU/ml (50µl) , indicating that the optimal multiplicity of infection for this experiment was 0.0009.


Titer of B. parapertussis 17903
Infected with Bacteriophage L1
3.7 x 10⁸ CFU/ml
5.6 x 10⁸ CFU/ml
1.0 x 10⁹ CFU/ml
1.1 x 10⁹ CFU/ml
2.1 x 10⁹ CFU/ml
2.7 x 10⁹ CFU/ml
Titer of Bacteriophage L1 Lysates (PFU/ml)
10¹²
10¹¹
10¹⁰
10⁹
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
Titer of Bacteriophage L1 used to Infect the Bacterial Cells (PFU/ml)

lysates of optimal titer include allowing the bacteriophage and host bacterial cells to pre-incubate at 4°C for 20 minutes prior to adding the molten soft-agar, incubating the soft-agar plates for ~ 24 hours and then flooding each soft-agar overlay with 3.0 ml of Lambda diluent. The optimal titers are acheived when the Lambda diluent bacteriophage lysate is removed without the soft-agar.

### 3.3 Broth Method of Bacteriophage Propagation :

In order to characterize the bacteriophage L1, sufficient quantities of bacteriophage had to be produced. The broth method of bacteriophage L1 propagation was more suited to mass production than was the soft-agar method. The broth method proved to be less time-consuming, manipulations easier to make, and the titers of the resulting bacteriophage lysates were higher and more consistent than those produced by the soft-agar method. The bacteriophage L1 was mass produced by the broth culture method to titers of 1 to 6 x $10^{10}$ PFU/ml.

### 3.3.1 Parameters Influencing the Titer of Bacteriophage L1 Lysate in Broth Culture.

The parameters influencing the final titers of the bacteriophage lysates produced from the infection of the bacterial host cells *B. parapertussis* 17903 with bacteriophage L1 were tested. The results are listed in sections 3.3.1.1 to 3.3.1.6.

#### 3.3.1.1 Production of Log-Phase Culture of Host Cells.

A method of predictably obtaining the logarithmic phase of a broth culture *B. parapertussis* 17903 to provide viable host cells for infection with the bacteriophage L1 was necessary. In order to produce bacteriophage lysates of high titer in broth culture, the bacterial host should be infected during the logarithmic phase of its growth (Adams

1959). A simple method used to obtain logarithmic phase cultures of *E. coli* is to inoculate a small volume (10 to 30 ml) of broth with a loopful of *E. coli* from an agar plate, incubate overnight to stationary phase, and inoculate a 100-fold volume of fresh broth with a sample from the overnight culture. A mid-logarithmic phase culture in most cases is available in 2 to 3 hours (Maniatis *et al.* 1982). *Bordetella parapertussis* 17903 can be conveniently grown on BGA plates and can remain viable in the humidified incubator at 37°C for at least 1 month. However, when a loopful of the bacterial growth approximately 3 days old was inoculated into several flasks with small volumes (20 ml) of TSB and incubated at 37°C with shaking, the flasks reached stationary phase at different times ranging from 20 to 30 hours (Figure 4). Equally important is the dramatic drop in the number of viable organisms once stationary phase had been reached (see section 3.3.1.2). Variation in the growth curves indicated a need for a standard sized inoculum of *B. parapertussis* 17903 so that the host cells could be harvested at the desired phase of growth as measured by optical density and after a standard period of incubation.

### 3.3.1.2 Correlating Optical Density of the Culture with Viable Counts

Using a standardized inoculum of 1 ml of *B. parapertussis* 17903 suspension (see section 3.3.1.1), a nephelometry flask with 100 ml of TSB was inoculated and incubated under standard conditions (see section 2.8.2). At time intervals, the optical density of the cultures was read at 540 nm and the viable counts determined by taking 1.0 ml samples and serially diluting in TSB. The dilutions were plated on TSA or BGA plates. The maximum number of viable cells per milliliter of culture was reached after 24 to 25 hours incubation at an optical density at 540 nm of 0.30 to 0.35. This corresponded to the logarithmic phase of the culture (Figure 5). Although the optical

**Figure 4.** Growth Curve of *B. parapertussis* 17903 in TSB without a Standardized Inoculum. Cultures of *B. parapertussis* 17903 in TSB, when inoculated with a loopful of bacterial growth from a three day old BGA plate showed that the mid-logarithmic phase of growth occured at times ranging from 20-30 hours. . The graph depicts the growth curves of three replicate bacterial cultures produced by plotting the optical density at 540 nm of the cultures over time.

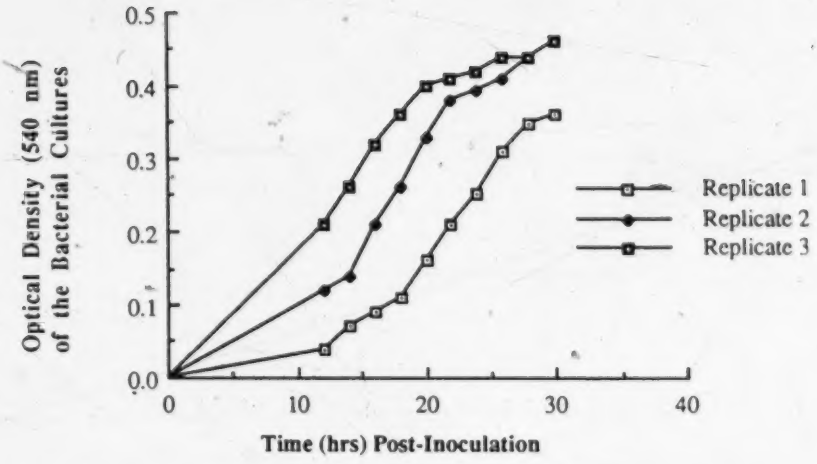
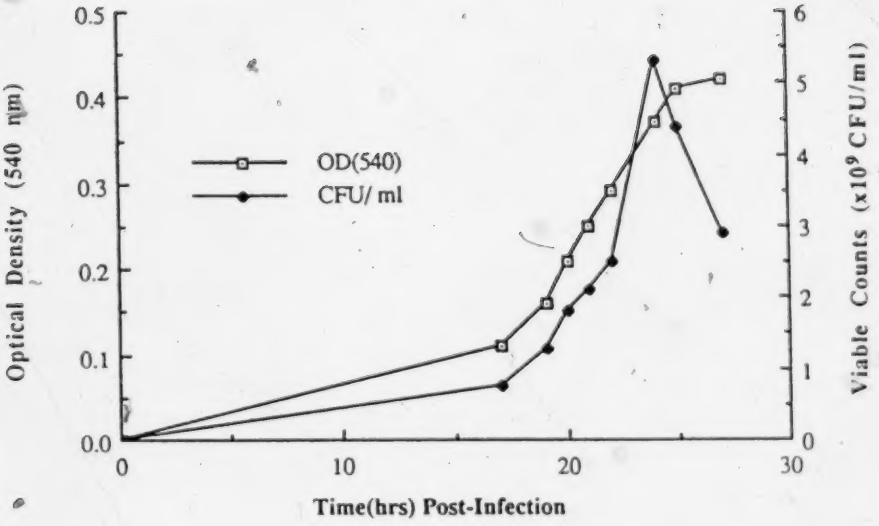

Optical Density (540 nm) of the Bacterial Cultures
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
Time (hrs) Post-Inoculation
Replicate 1
Replicate 2
Replicate 3

**Figure 5.** Growth Curve of *B. parapertussis* 17903 in TSB with a Standardized Inoculum. The growth curve of *B. parapertussis* 17903 produced when 100 ml of TSB was inoculated with a standardized bacterial inoculum (1 ml of a bacterial suspension with an optical density at 540 nm of 0.2). The figure shows a typical time course of *B. parapertussis* 17903 cultures when inoculated with this standardized inoculum, correlating optical density of the culture with number of viable bacterial cells produced over time. The mid-logarithmic phase of bacterial growth was reached after 24 to 25 hours of incubation with shaking at 37°C. A sharp decrease in the viable counts occurs at stationary phase.


Optical Density (540 nm)
0.5
0.4
0.3
0.2
0.1
0.0
OD(540)
CFU/ ml
Viable Counts (x10⁹ CFU/ml)
6
5
4
3
2
1
0
0
10
20
30
Time(hrs) Post-Infection

density of the cultures continued to increase to 0.6, the viable counts of organisms plummeted in a relatively short time after the maximal viable counts had been reached. Because of the short stationary phase, the host bacterial cells were harvested when the culture was closer to 0.3 than 0.35 whenever possible.

### 3.3.1.3 Adsorption of Bacteriophage L1 at Various Multiplicities of Infection and Temperatures.

Previous work with propagation of the bacteriophage L1 on the host cells *B. parapertussis* 17903 using the soft-agar method had indicated that the titers of the resulting lysates were higher when the bacteriophage L1 was pre-incubated with the bacterial hosts in the cold room (~ 4°C) rather than at room temperature or 37°C. Hypothetically, the higher lysate titers could have resulted from a more efficient adsorption of the bacteriophage L1 to the host cells, or possibly that less degradation of the infecting bacteriophage L1 occurred at 4°C as compared to 37°C (assuming degradation was occurring).

The following experiment was designed to determine if the bacteriophage L1 would adsorb more efficiently to the host cells at 4°C or at 37°C. A suspension of *B. parapertussis* 17903 was prepared by scraping the bacterial growth from 36 hour BGA plates, resuspending it in 40 ml TSB and adjusting the suspension to an optical density of ~0.3 at 540 nm. A 5.0 ml volume of the adjusted bacterial suspension was added to 6 sterile screw-capped tubes. The adjusted bacterial suspension was diluted 1 in 10 by adding 0.5 ml aliquots of the adjusted bacterial suspensions to 6 more sterile screw-capped tubes containing 4.5 ml of TSB. The twelve tubes were then divided into two sets of six, each containing three tubes of the adjusted bacterial suspension and three tubes of the diluted adjusted bacterial suspensions. One set of 6 tubes was placed in a

37°C waterbath and the other set of 6 tubes were placed in the cold room at ~4°C and allowed to equilibrate for 10 minutes. The bacteriophage lysate used was a filter sterilized lysate with an initial titer of ~$10^9$ PFU/ml. Two further suspensions of the bacteriophage L1 lysate were made, the first by adding 0.2 ml of the bacteriophage L1 lysate to 20 ml of TSB (1: 100) and the second, a 1: 10000 dilution was made by adding 0.2 ml of the 1:100 diluted L1 bacteriophage suspension to 20 ml of TSB. The bacteriophage L1 suspensions were transferred into twelve screw-capped tubes in 5.0 ml aliquots. The twelve screw-capped tubes containing bacteriophage L1 were divided into two sets of six tubes. Each set contained two tubes of the bacteriophage L1 lysate undiluted, at a 1:100 dilution and at a 1:10000 dilution. One set of the bacteriophage suspensions was placed at 4°C and the other at 37°C at the same time as the bacterial suspensions to allow the temperatures of the two types of suspensions to equilibrate. The bacteriophage dilutions were made prior to the bacterial dilutions as the bacteriophage are stable when stored at 4°C in TSB whereas the *B. parapertussis* 17903, being living organisms are more likely to be affected by the delays during preparation of the bacteriophage L1 dilutions. After the equilibration time, 5.0 ml of the various bacteriophage suspensions were mixed with 5 ml of the bacterial suspensions in the following design :

Tube #1: adjusted bacterial suspension (~4 x $10^9$ CFU/ml)+ undiluted bacteriophage lysate ($10^9$ PFU/ml)

Tube #2: adjusted bacterial suspension (~4 x $10^9$ CFU/ml)+ 1:100 dilution of bacteriophage lysate ($10^7$ PFU/ml)

Tube #3: adjusted bacterial suspension (~4 x $10^9$ CFU/ml) + 1:10000 dilution bacteriophage lysate ($10^5$ PFU/ml)

Tube #4: diluted bacterial suspension (~4 x $10^8$ CFU/ml) + undiluted bacteriophage lysate ($10^9$ PFU/ml)

Tube #5: diluted bacterial suspension (~4 x $10^8$ PFU/ml)+ 1:100 dilution of bacteriophage lysate ($10^7$ PFU/ml)

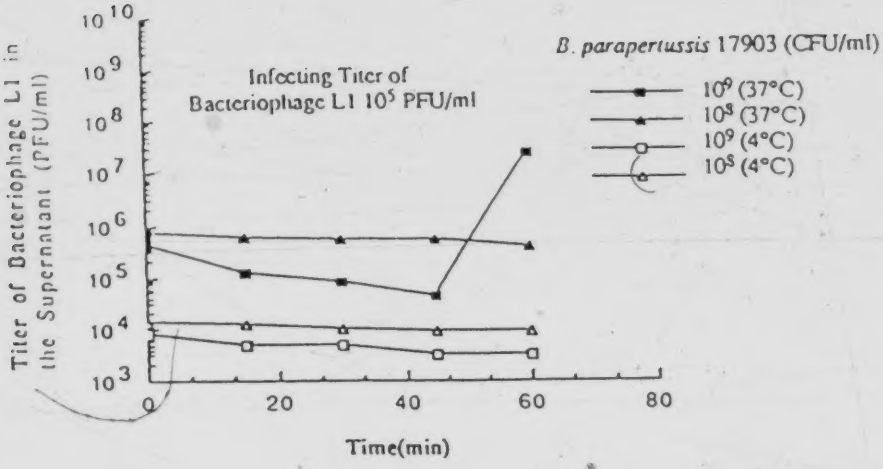
Tube #6: diluted bacterial suspension (~4 x $10^8$ PFU/ml)+ 1:10000 dilution of bacteriophage lysate ($10^5$ PFU/ml)

One set of tubes was at 37°C and the other at 4°C. A 200 µl sample of the mixed solutions was taken at the time of mixing (t=0) and placed in an Eppendorf tube. The Eppendorf tubes were centrifuged at 12000 x g for 5 minutes to pellet the bacteria, 100 µl of the supernatant was removed, serially diluted in TSB and the titer of the bacteriophage determined by spotting 10 µl portions of the diluted samples on indicator plates (see section 2.5). Samples were taken at t = 15, 30, 45, and 60 minutes. The 10 ml bacteria-bacteriophage mixture was used to minimize the effect of reducing total volume of the test bacteriophage and host bacterial cells as a result of sampling.

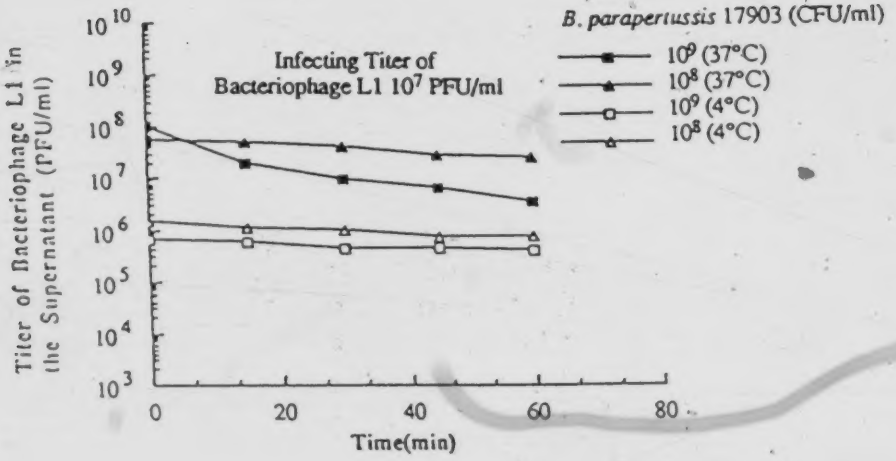
Certain trends are apparent from the graphs of the titer of bacteriophage L1 in the supernatant over time at either 4°C and 37°C (Figure 6). The best adsorption of bacteriophage L1 to the host cells *B. parapertussis* 17903 was at 4°C. The bacteriophage L1 appeared to adsorb very quickly at 4°C as compared to 37°C. At t=0, there was a 100 fold lower titer of bacteriophage L1 in the supernatant as compared to the tubes at 37°C. Even after 60 minutes at 37°C, the bacteriophage titers of the supernatant were still at a 10 fold higher value than those at t =0 at 4°C. The decrease in titers of the bacteriophage at 37°C was not due to deterioration of the bacteriophage as a tube with bacteriophage L1 lysate incubated in the 37°C waterbath for the same time the test bacteriophage L1 suspensions did not show a decrease in titer over the duration of the experiment. It is also apparent that after 30 minutes of incubation at

**Figure 6.** Adsorption of Bacteriophage L1 to *B. parapertussis* 17903 cells at 4°C and 37°C. Three titers of bacteriophage L1 ($10^9$, $10^7$, $10^5$ PFU/ml)were used to infect two different concentrations of *B. pertussis* 17903 (~4 x $10^9$ and 4 x $10^8$ CFU/ml) at two temperatures (4°C and 37°C). Samples of the mixtures were centrifuged to remove the bacterial cells with the adsorbed bacterial cells and the supernant assayed by the plaque assay to determine the titer of unbound bacteriophage L1 remaining in the supernatant. Adsorption of bacteriophage L1 to the bacterial cells over a one hour observation period was determined from the titer of bacteriophage in the supernants. **Figure 6a** shows the adsoption of bacteriophage L1 ($10^5$ PFU/ml) to bacterial cells (bacterial concentration = ~4 x $10^9$ and 4 x $10^8$ CFU/ml) at 4°C and 37°C. **Figure 6b** shows adsorption of bacteriophage L1 ($10^7$ PFU/ml) to bacterial cells (bacterial concentration = ~4 x $10^9$ and 4 x $10^8$ CFU/ml) at 4°C and 37°C. **Figure 6c** shows adsorption of bacteriophage L1 ($10^9$ PFU/ml) to bacterial cells (bacterial concentration = ~4 x $10^9$ and 4 x $10^8$ CFU/ml) at 4°C and 37°C. In all cases, bacteriophage L1 adsorbed to the bacterial cells more efficiently at 4°C than at 37°C.

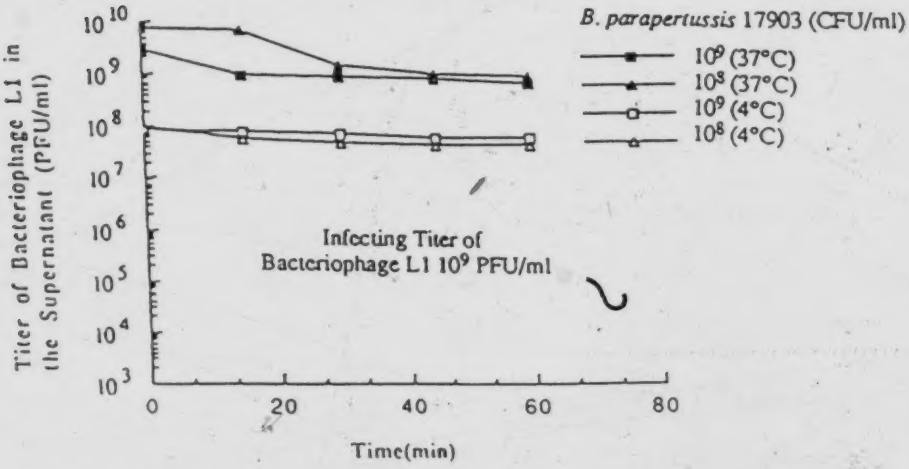
6a)


Titer of Bacteriophage L1 in the Supernatant (PFU/ml)
Infecting Titer of Bacteriophage L1 10^5 PFU/ml
B. parapertussis 17903 (CFU/ml)
10^9 (37°C)
10^8 (37°C)
10^9 (4°C)
10^8 (4°C)
Time(min)

6b)


Titer of Bacteriophage L1 in the Supernatant (PFU/ml)
Infecting Titer of Bacteriophage L1 10^7 PFU/ml
B. parapertussis 17903 (CFU/ml)
10^9 (37°C)
10^8 (37°C)
10^9 (4°C)
10^8 (4°C)
Time(min)

6c)


Titer of Bacteriophage L1 in the Supernatant (PFU/ml)
Infecting Titer of Bacteriophage L1 10^9 PFU/ml
B. parapertussis 17903 (CFU/ml)
10^9 (37°C)
10^8 (37°C)
10^9 (4°C)
10^8 (4°C)
Time(min)

4°C, not many more bacteriophage adsorbed to the bacterial cells. In all cases, mixture with the greater amount of bacterial cells produced a lower titer of bacteriophage in the supernatant which would be expected considering that there would be a greater number of bacteriophage receptors present, but interestingly, not the 10-fold difference as one might expect from the difference in bacterial concentrations. It appeared that the temperature had an effect on adsorption of the bacteriophage L1 to the bacterial cells and that the 20 to 30 minute pre-incubation of the bacteriophage L1 with the host bacterial cells was sufficient time for adsorption.

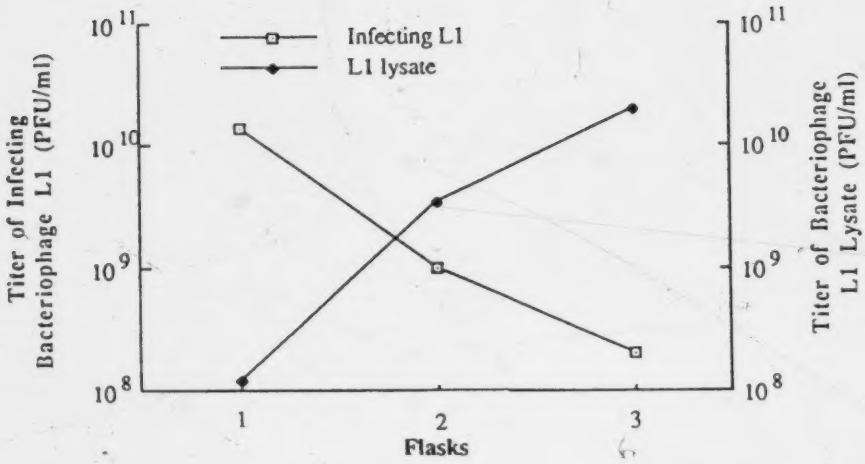
The successful protocol for propagation of broth cultures of bacteriophage L1 was based on the protocol for the propagation of bacteriophage λ which called for the infection of pelleted bacterial cells to aid in adsorption of bacteriophage λ to the host cells (Maniatis *et al.* 1982). An experiment was conducted to determine if *B. parapertussis* 17903 cells had to be pelleted to be optimally infected infected or if the bacteria could be suitably infected with the bacteria in suspension. Two twenty milliliter aliquots of log phase *B. parapertussis* 17903 were taken. One aliquot was centrifuged for 10 minutes at 8 000 x g, the supernatant decanted and the pelleted bacterial cells suspended in 1 ml of bacteriophage L1 lysate and allowed to pre-incubate at 4°C for 20 minutes. The other aliquot was placed at 4°C while the first aliquot was being pelleted to equilibrate the temperature of both aliquots in preparation for bacteriophage L1 adsorption. The unpelleted bacterial suspension was infected with 1 ml of the same bacteriophage lysate that was used to infect the pelleted bacteria. The bacteriophage and bacteria in both aliquots were allowed to pre-incubate for 20 minutes at 4°C, then transferred to two different 2-L Erlenmeyer flasks each containing 500 ml of TSB and incubated as per the protocol for the broth culture method (see section 2.8.2). The resulting titers of the two lysates showed the pelleted bacteria produced a

titer of 2 x $10^{10}$ PFU/ml while the unpelleted bacteria produced a titer of 3 x $10^{7}$ PFU/ml (Data not shown). The most efficient means of infecting the host cells, therefore, was by first pelleting them.

### 3.3.1.4 Determination of the Optimal Multiplicity of Infection:

The host cells, *B. parapertussis* 17903 were prepared as described in the broth method for bacteriophage L1 propagation (see section 2.8.2). An experiment was conducted to determine if a low multiplicity of infection was optimal for the broth method. Various titers of bacteriophage were used to infect the pelleted host cells with the volume of bacteriophage lysate kept constant at 1 ml. The host cells were harvested from the logarithmic phase of the broth culture of *B. parapertussis* 17903 which correlated with the highest viable count ($OD_{540}$ ~0.3). Twenty milliliter portions of the culture were used because of the volume of available Corex tubes and the convenience of using them. The titers of the resulting bacteriophage lysates were determined by the plaque assay method (see section 2.5) (Figure 7). The optimal multiplicity of infection for the broth method of propagation was found to be 0.002-0.005. Although an end point with the absolute optimal multiplicity of infection was not obtained, the optimal multiplicity of infection for this system was attained. The maximum number of cells to be pelleted in the 20 ml Corex tubes were defined by the maximum viable cells in mid-logarithmic phase and the maximum volume of broth culture that could be added to the Corex tubes. Any further increase in pelleted cells would require use of larger centrifuge vessels. The decrease of titer added to the pelleted cells to $10^{5}$ PFU/ml had resulted in resultant bacteriophage lysates of approximately $10^{9}$ PFU/ml in other experiments. The volume of TSB that the cells were being added to was only 500 ml in a 2-liter Erlenmeyer to ensure adequate aeration conditions on the shaker incubator. In

**Figure 7.** Optimal Multiplicity of Infection for the Broth Method of Bacteriophage L1 Propagation. To determine the optimal multiplicity of infection for the broth method of bacteriophage L1 propagation, the standard bacterial inoculum (20 ml of mid-logarithmic phase *B. parapertussis* 17903) was pelleted by centrifugation and the drained pellet infected with 1.0 ml of a bacteriophage L1 lysate. Three replicate bacterial pellets were incubated at 4°C with bacteriophage L1 lysate for 20 to 30 minutes. Titers of $10^{10}$ PFU/ml, $10^{9}$ PFU/ml and $10^{8}$ PFU/ml were tested. After adsorption, the suspension was added to 500 ml of TSB and incubation at 37°C for 24 hours. The resulting titer of bacteriophage L1 lysates revealed that for the broth method of propagation, the optimal multiplicity of infection approaches 0.002-0.005.


Infecting L1
L1 lysate
Titer of Infecting Bacteriophage L1 (PFU/ml)
Titer of Bacteriophage L1 Lysate (PFU/ml)
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
1
2
3
Flasks

the future, further studies may show that a different multiplicity of infection is more suitable if the conditions of growth of the bacteriophage L1 are changed. Such effects are well-documented in the literature (Adams 1954). For the method described for the broth culture propagation of bacteriophage L1, the best multiplicity of infection was found to range from 0.002-0.005. This protocol provided sufficient titers of bacteriophage for the studies of bacteriophage L1 DNA, therefore further investigation of multiplicity of infection was not pursued.

### 3.3.1.5 Determination of Optimal Incubation Time:

A time course experiment was done in order to determine when the maximum titer of bacteriophage was present and if leaving the culture too long at 37°C would affect the titer of the lysate. Three replicate mid-logarithmic trypticase soy broth cultures of *B. parapertussis* 17903 were infected as described in the broth method of bacteriophage propagation using the optimal multiplicity of infection. At various time intervals a 1 ml sample of the infected bacterial cultures was removed, the bacteria pelleted by centrifuging in a benchtop centrifuge at 12 000 x g, a 100 µl sample of the supernatants serially diluted in TSB and the bacteriophage titer determined by the plaque assay (see section 2.5). The optimal time for the harvest of the bacteriophage lysate appeared to be 27 to 29 hours post-infection (Figure 8). There was a loss in titer in the lysate observed after 32 hours.

**3.3.1.6. SUMMARY:** Conditions for the production of optimal titers of bacteriophage L1 by the broth method are as follows: infection of a mid-logarithmic culture of *B. parapertussis* 17903 (~ 2 x $10^{10}$ CFU/ml) , pelleted by centrifugation and resuspended in bacteriophage L1 lysate at a multiplicity of infection of 0.002-0.005 bacteriophage per bacterium. The bacteriophage L1 were allowed to adsorb to the host

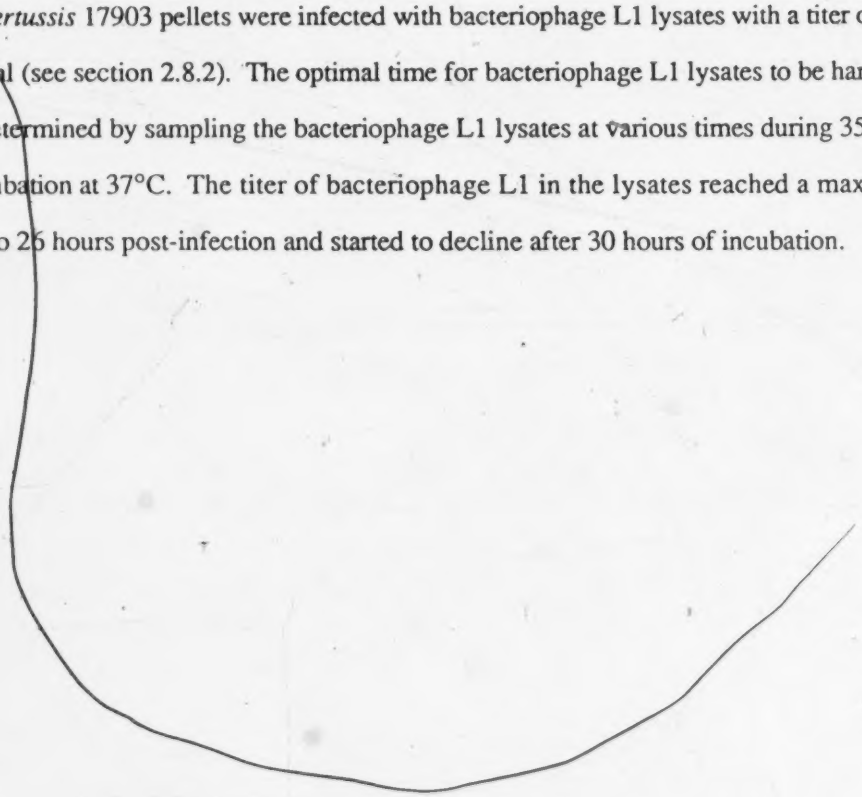
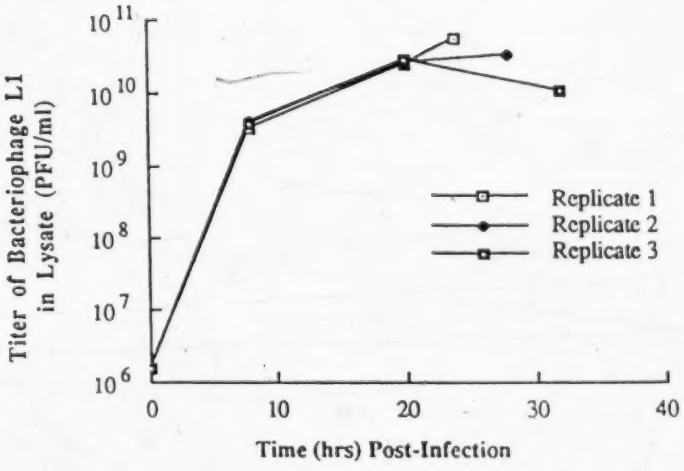


**Figure 8.** The Optimal Time for Harvest of Bacteriophage L1 Lysates. Three replicate *B. parapertussis* 17903 pellets were infected with bacteriophage L1 lysates with a titer of $10^8$ PFU/ml (see section 2.8.2). The optimal time for bacteriophage L1 lysates to be harvested was determined by sampling the bacteriophage L1 lysates at various times during 35 hours of incubation at 37°C. The titer of bacteriophage L1 in the lysates reached a maximum titer at 24 to 26 hours post-infection and started to decline after 30 hours of incubation.


Titer of Bacteriophage L1
in Lysate (PFU/ml)
10 11
10 10
10 9
10 8
10 7
10 6
0
10
20
30
40
Time (hrs) Post-Infection
Replicate 1
Replicate 2
Replicate 3

bacterial cells for 20 to 30 minutes at 4°C, then incubated with shaking at 37°C and the bacteriophage L1 lysate harvested after 24 to 26 hours. The temperature of the bacteriophage and host cells appeared to affect the adsorption of the bacteriophage to the bacterial hosts.

### 3.4 Morphology of Bacteriophage L1:

All electron microscopy was conducted on the concentrated bacteriophage L1 lysates. The electron micrographs of the bacteriophage L1 revealed that the bacteriophage had an icosahedral head with a collar, a contractile tail, and a tail plate assembly with four projections which became visible on contraction of the bacteriophage tail (Plate 1). The dimensions of the bacteriophage were as follows : head, 56 nm in diameter; tail, 77 x 15 nm in the uncontracted form and 25 x 16 nm in the contracted form. Based on these morphological features, the bacteriophage L1 belonged in the Bradley group A1 and appeared to belong to the I.C.T.V. family of Myoviridae.

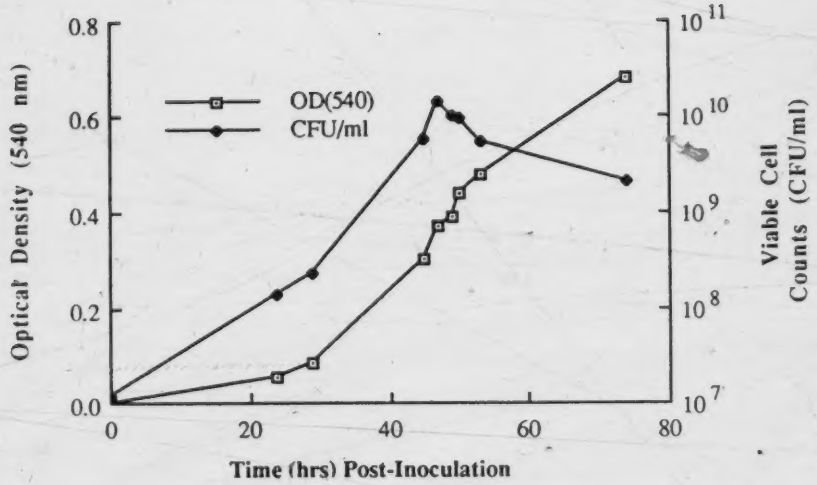
### 3.5 Testing for Bacteriophage L1 Plaque Production on strains of *B. pertussis* .

#### 3.5.1 Growth Curves of *B. pertussis* Strains and Efficiency of Plating of BGA, SSA and CSM.

Initial host range showed that the bacteriophage L1 did not form plaques on *B. pertussis* strains, however, the underlying agar used was BGA, an opaque agar with the inherent difficulty of detecting plaque formation. In addition, little attention was paid to details concerning the temperature of adsorption or growth phase of the bacterial host. The following experiment was done to determine which clear agar, SSA or

**Plate 1**: Electronmicrographs of bacteriophage L1 as determined by negative staining with ammonium molybdate. The bacteriophage L1 is shown in both its contracted and non-contracted forms, and appears to be bound to fragments of *B. parapertussis* 17903 outer membranes.

**Figure 9.** The Growth Curve for *B. pertussis* BB103 in SSB. A standardized bacterial inoculum of 1.0 ml of bacterial suspension adjusted to an optical density at 540 nm of 0.2 was used to inoculate 100 ml of SSB. The inoculated SSB was incubated with shaking at 37°C. The optical density of the culture was determined at 540 nm and samples of the culture taken at various times to determine the numbers of viable organisms. Viable counts were conducted on BGA plates. The mid-logrithmic phase of growth was reached after approximately 40 hours of incubation. Similar growth curves were generated for *B. pertussis* strains Sak$^+$, 3779$^-$, 3779$^+$, and 18323. The short stationary phase was apparent in the growth curves of *B. pertussis* strains as it was in the *B. parapertussis* strains. Note that *B. pertussis* took 40 hours to reach mid-log phase whereas *B. parapertussis* took 20 hours to reach mid-log phase (Figure 5).


Optical Density (540 nm)
0.8
0.6
0.4
0.2
0.0
OD(540)
CFU/ml
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
$10^{7}$
Viable Cell Counts (CFU/ml)
0
20
40
60
80
Time (hrs) Post-Inoculation

CSM, could most efficiently support the growth of the *B. pertussis* strains. Growth curves of the *B. pertussis* strains were done in order to determine which optical density correlated with the maximum viable bacteria and to see if the stationary phase was as short and the death phase as dramatic as found with *B. parapertussis* cultures (see Figure 5). A growth curve similar to the one done for *B. parapertussis* 17903 was constructed for strain BB103 of *B. pertussis* (Figure 9). The protocol for growing the strains of *B. pertussis* was the same as that for *B. parapertussis* 17903 except that *B. pertussis* was grown in SSB. The optical density at 540 nm of the SSB cultures of *B. pertussis* were monitored over time and 100 µl samples were taken, serially diluted in 900 µl of SSB and 50 µl samples of the serial dilutions plated on BGA, SSA, and CSM agars. The colony counts on the clear media (CSM and SSA) were compared to those obtained on BGA plates, BGA being a standard medium used to support the maximal growth of a wide range of *B. pertussis* strains. The equation used to determine the efficiency of plating was as follows: :

$$\frac{\text{CFU/ml on test medium}}{\text{CFU/ml on BGA}} \times 100 = \%\ \text{efficiency of plating.}$$

BGA best supported the best growth of most *B. pertussis* strains, however, either SSA or CSM supported the bacterial growth to approximately 80 to 90% efficiency (Table 4). Also, the colonies grew slightly larger on the BGA plates as compared to the CSM and SSA plates. Therefore, either CSM or SSA could be used for the support agar in the soft-agar overlays.

### 3.5.2 Plaque Formation on Strains of *B. pertussis* .

Log phase cultures of *B. pertussis* were infected with bacteriophage L1. An SSB culture of *B. pertussis* was incubated at 37°C in a shaker incubator until the absorbance of the culture at 540 nm was ~ 0.3. Aliquots of the culture (0.40 ml) were

**Table 4**: Percent Efficiency of Plating for strains of *B. pertussis* and *B. parapertussis* as determined for Bordet-Gengou agar (BGA), cyclodextrin-solid medium (CSM) and Stainer-Scholte agar (SSA).

| Bacterial strain | Media | | |
|---|---|---|---|
| | BGA | CSM | SSA |
| *B. pertussis* 3779 + | 100 | 106 | 98 |
| *B. pertussis* 3779 - | 100 | 87 | 92 |
| *B. pertussis* BB103 | 100 | 83 | 89 |
| *B. pertussis* 18323 | 100 | 88 | 84 |
| *B. pertussis* 2753 | 100 | 92 | 87 |
| *B. parapertussis* 17903 | 100 | 93 | 95 |

dispensed into Eppendorf tubes, centrifuged in a benchtop Eppendorf centrifuge at 12 000 x g for 5 minutes to pellet the bacteria, and the supernatant decanted. The pelleted cells were suspended in bacteriophage L1 lysate of known titer. The infected cells were allowed to incubate at 4°C for 20 minutes and plated by the soft-agar method using CSM and SSA plates for the bottom agar and the PBS-sloppy agar for the soft-agar overlay. The soft-agar plates were incubated at 37°C in a humidified incubator for 1 week and the plates were observed for plaques every 24 hours starting from the the time of infection.

As an alternative means of infecting *B. pertussis*, soft-agar overlays of *B. pertussis* were prepared as indicator plates. The media used was CSM and SSB. The bacteriophage L1 was spotted onto the overlays prior to incubation at 37°C and after 24 hours of incubation at 37°C. Adsorption of the bacteriophage in both cases occurred at room temperature. The plates were observed for plaque formation at 24 hour intervals for 1 week. Using these same techniques that produced plaquing on *B. parapertussis*, the bacteriophage L1 did not produce any plaques on any of the *B. pertussis* strains tested. The strains of *B. pertussis* (BB103, 18323, 3779+, 3779-, 2753, SAK+, SAK-) tested were chosen because of their phenotypic similarity to *B. parapertussis* strains. The positive control used was *B. parapertussis* 17903 carried through the same procedures as the *B. pertussis* strains. Uninfected cultures of *B. pertussis* and *B. parapertussis* 17903 served as negative controls to ensure the viability of the bacteria.

### 3.6 Concentration of Bacteriophage L1 Lysates:

In order to see visible bands on the cesium chloride gradients, the crude bacteriophage lysates had to be concentrated. The efficiency of the concentration

procedure (see section 2.9 ) was measured by infectivity of the bacteriophage lysate or the number of plaque forming units per milliliter of bacteriophage suspension (see Table 5). This is an appropriate measure since only infective and presumably intact bacteriophage were expected to form the lower band in the cesium chloride gradients (Figure 10). Best results for the bacteriophage L1 purification on the cesium chloride gradients were obtained if the polyethylene precipitated bacteriophage concentrates (pelleted bacteriophage particles resuspended in TSB) were applied to the cesium chloride gradients as soon as possible after they were precipitated. Even storage at 4°C for 8 hours or overnight reduced the infectivity of the bacteriophage suspension by 45% (Data not shown). Also on visual inspection of the cesium chloride gradients, the width of the lower band containing the intact bacteriophage was decreased and the upper band was increased if there was a substantial delay in adding the concentrated bacteriophage to the gradients.

**3.7 Sensitivity of Bacteriophage L1 to Environment :** The effect of the NaCl, CsCl, $CHCl_3$, and heat on the infectivity of the bacteriophage L1 over time were monitored :

**3.7.1. 1 M NaCl** : The titer of the bacteriophage lysates in broth diminished by approximately 100-fold in the first 24 hours even if it was stored at 4°C. The lysates were not monitored after the 24 hour time period for possible further decline in the bacteriophage titers. This observation was important because the bacteriophage L1 was concentrated by polyethylene glycol and 1 M NaCl precipitation.

**3.7.2. 1M CsCl:** The titer of the bacteriophage L1 lysates remained stable in 1 M CsCl for 1 week (period of observation). Generally, the purified bacteriophage lysates

**Figure 10**. Purification of Bacteriophage L1, T4, and λ on Cesium Chloride Step Gradients. Bacteriophage L1, T4, and λ were centrifuged on cesium chloride step gradients. The gradients were fractioned into approximately 10 drop fractions. A one hundred microliter sample of each fraction was then diluted 1 in 10 with Lambda diluent and the absorbance of the diluted fraction determined at 260 nm wavelength. The figure reveals the fractions of the step gradient which usually contain the bacteriophage. There are usually two peaks in each gradient, with the peak closer to the bottom of the gradient presumably being intact bacteriophage particle and the upper peak being incomplete bacteriophage particles. For characterization of the bacteriophage L1, only the lower banding bacteriophage particles were used.

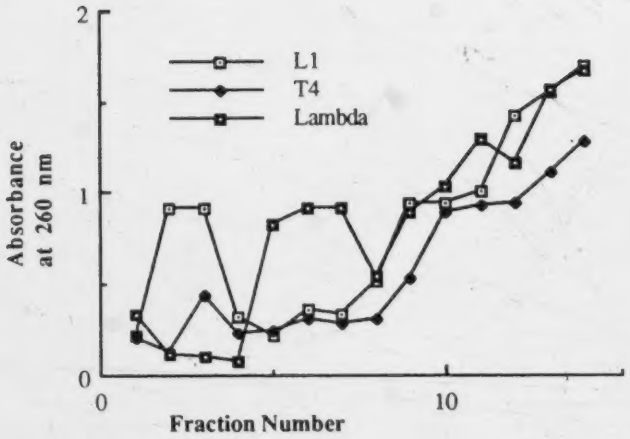

L1
T4
Lambda
Absorbance at 260 nm
2
1
0
0
10
Fraction Number

**Table 5 : Efficiency of Concentration of Bacteriophage L1** The efficiency of concentration of the bacteriophage L1 on cesium chloride step gradients was determined by measuring the titer of the bacteriophage L1 and the volume of the bacteriophage L1 suspension when removed from the gradient.

| | Titer (PFU/ml) | Volume (ml) | Total PFU | % Recovery |
|---|---|---|---|---|
| Lysate 1 | $1.4 \times 10^{10}$ | 120 | $1.7 \times 10^{12}$ | 100 |
| Lysate 2 | $1.0 \times 10^{10}$ | 120 | $1.2 \times 10^{12}$ | 100 |
| PEG ppt 1 | $9.0 \times 10^{10}$ | 3 | $2.7 \times 10^{11}$ | 16 |
| PEG ppt 2 | $9.1 \times 10^{10}$ | 3 | $2.73 \times 10^{11}$ | 23 |
| Lysate 3 | $7.3 \times 10^{9}$ | 120 | $8.76 \times 10^{11}$ | 100 |
| PEGppt 3 with NaCl | $8.9 \times 10^{10}$ | 3 | $2.6 \times 10^{11}$ | 30 |
| PEGppt 3 without NaCl | $1.3 \times 10^{11}$ | 3 | $3.9 \times 10^{11}$ | 45 |

Abbreviations : Lysate = crude bacteriophage lysate harvested from ther broth method of propagation (section 2.8.2), PEGppt = polyethylene glycol precipitated bacteriophage suspended in TSB (section 2.9), with NaCl = bacteriophage L1 polyethylene glycol precipitated with NaCl, without NaCl = bacteriophage L1 polyethylene glycol precipitated without the addition of NaCl.

were dialysed against a 0.1M Tris-HCl buffer (pH 7.5) immediately following removal of the bacteriophage from the cesium chloride gradients.

**3.7.3. Chloroform** : The titer of the bacteriophage L1 was unaffected by either the 1 to 2 drops of chloroform added to the 50 to 200 ml samples of bacteriophage lysate or by the volume-for-volume extraction (see section 2.17) of the polyethylene glycol from precipitated bacteriophage lysates.

**3.7.4 Temperature:** When bacteriophage lysates were left at room temperature in TSB, the titer of the bacteriophage decreased approximately 10 fold in a 24 hour period.

**3.8 Purification of Bacteriophage L1 on Cesium Chloride Gradients** :

The bacteriophage L1 formed a band at a refractive index of ~ 1.38 which corresponded to a density of approximately 1.49 g/ml. The cesium chloride gradients were fractionated by removing the gradient with a peristalsic pump, each fraction was diluted 1 in 10 in Lambda diluent and the absorbances read at $A_{260}$ and $A_{280}$. Two peaks were observed. The top peak had a refractive index of 1.3580 and the bottom band of presumably intact bacteriophage at a refractive index of 1.38 (Figure 10). During the purification of the bacteriophage λ, the bottom band of intact bacteriophage particles was removed from the cesium chloride gradients (Maniatis *et al.* 1982). For consistancy in the characterization of the bacteriophage L1, the lower band from the cesium chloride gradient was also routinely used.

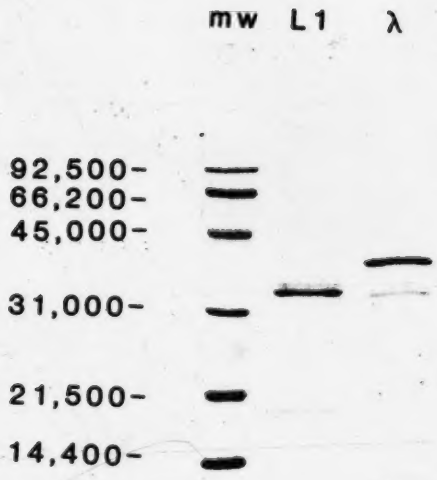
### 3.9 Protein Pattern

The bacteriophage L1 was radiolabelled to label the surface proteins and the bacteriophage was TCA precipitated to remove the bacteriophage particles from the unincorporated $^{125}I$. The result was an acidic pellet (see section 2.13). Unlabelled bacteriophage L1 ($A_{260}$ = 6.0, 100 µl ) was added to the acidic pelleted bacteriophage L1 and the pellet was neutalized with very small amounts of 10 N NaOH. The same procedure was used for the samples of bacteriophage λ and T4. Bromophenol blue in the Laemmli digestion mix was used to determine when the solution was no longer acidic as it changed from yellow to blue. All samples were loaded onto the gels within 15 minutes of adding the base to further minimize the pH effects. The major proteins of the bacteriophage L1, as viewed by Coomassie brilliant blue staining and autoradiography had approximate molecular weights of 35 KD, 33 KD, 18.5 KD and 17 KD. An extremely faintly staining band with an approximate molecular weight of 30 KD was observed in some of the Coomassie stained bacteriophage preparations (see Plate 2a). The major proteins found in the bacteriophage λ preparation had approximate molecular weights of 55 KD, 47 KD, 42 KD, and 13 KD. These compared favorably to the published molecular weights of the major external head proteins which are 56.0 KD, 46.5 KD, 43.5 KD, and 31.0 KD (Laemmli 1970). A minor 31.0 KD band was also seen in the bacteriophage λ preparation. The major bands found in $^{125}$ iodinated bacteriophage T4 preparations had approximate molecular weights of 50 KD and 24.8 KD. The pattern produced by the bacteriophage L1 was distinct from that produced by bacteriophage T4 and λ (Plate 2).

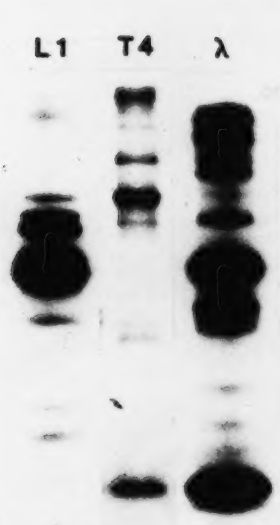
Coomassie brilliant blue staining revealed only the major proteins present in the samples of all three bacteriophage. The autoradiographs of the Coomassie brilliant blue

**Plate 2 :** The Protein Patterns of Bacteriophage L1, T4, and λ.

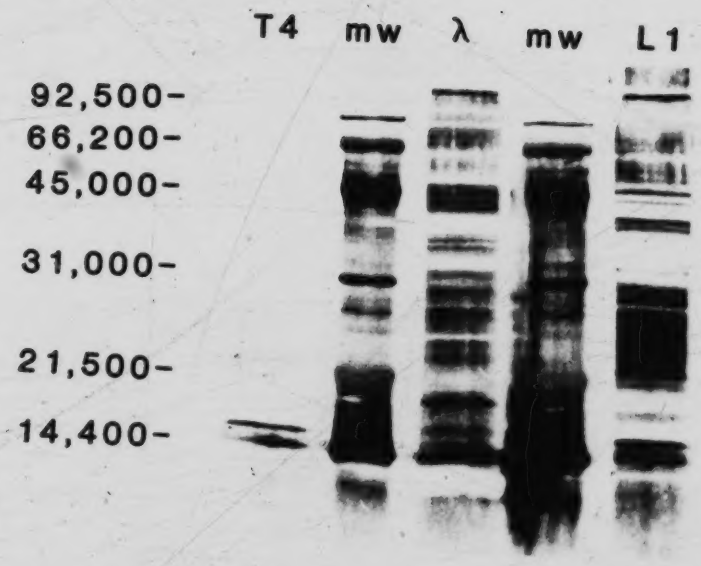
2a. The Protein Patterns of Bacteriophage L1, T4, and λ. Coomassie brilliant blue staining of bacteriophage L1 and λ following SDS-polyacrylamide gel electrophoresis.


mw
L1
λ
92,500-
66,200-
45,000-
31,000-
21,500-
14,400-

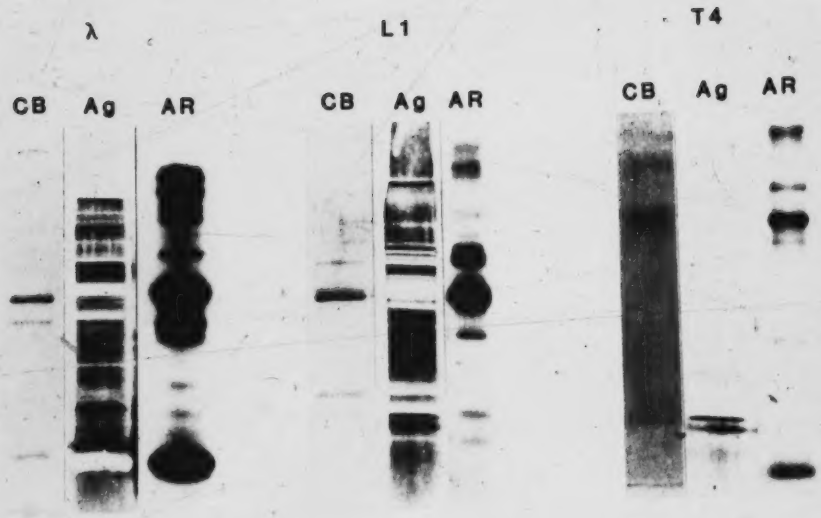
2b. The Protein Patterns of Bacteriophage L1, T4, and λ. Autoradiographs of bacteriophage L1, T4, and λ surface-labelled with $^{125}I$ and subjected to SDS-polyacrylamide gel electrophoresis.


L1
T4
λ

2c. The Protein Patterns of Bacteriophage L1, T4, and λ. Silver staining of bacteriophage L1, T4, and λ following SDS-polyacrylamide gel electrophoresis.


T4
mw
λ
mw
L1
92,500-
66,200-
45,000-
31,000-
21,500-
14,400-

**2d**. The Protein Patterns of Bacteriophage L1, T4, and λ. Composite pictures comparing the Coomassie brilliant blue stained, silver stained and autoradiographs of bacteriophage L1, T4, and λ. (abbreviation: CB=Coomassie brilliant blue stained, Ag=silver stained, AR=autoradiograph, T4=bacteriophage T4, λ=bacteriophage Lambda, L1=bacteriophage L1)


λ
CB
Ag
AR
L1
CB
Ag
AR
T4
CB
Ag
AR

stained gels also showed the same major proteins as determined by the relative intensities of the bands on the autoradiograph (see Plate 2b).

Silver staining revealed several more proteins than did the Coomassie brilliant blue staining however, the major proteins shown in the Coomassie brilliant blue stain did not stain with the silver stain but rather showed up as clear negatively-stained bands only when the background of the gel was allowed to overdevelop.

The $^{125}I$ surface labelling of the bacteriophage L1 revealed that all the proteins detected by the silver stain appear in the autoradiograph. This suggested that the proteins visualized in the silver stain and the Coomassie brilliant blue stain are proteins with an exposure to the exterior of the bacteriophage. No strictly interior proteins were detected by these methods.

**3.10 Burton Diphenylamine Assay Results :**

The electron micrographs of the bacteriophage L1 revealed the bacteriophage to be a tailed, contractile bacteriophage (see section 3.4). Of the bacteriophage reported, almost 98% of tailed, contractile bacteriophage contained double-stranded DNA (Ackermann 1987). Therefore, the diphenylamine assay for detection of DNA was done on a sample of bacteriophage L1 that had been purified on a cesium chloride gradient and the salt removed by dialysis against 0.1 M Tris-HCl buffer pH 7.5. The purified bacteriophage L1 was diluted 1:10 in Lambda diluent to determine an accurate $A_{260}$ reading to estimate the amount of nucleic acid. The reading was 0.6. Using the conversion factor that 1 $OD_{260}$ = 50 µg DNA/ml, the amount of nucleic acid estimated was 300 µg/ml in the bacteriophage sample. A 50 and 100 µl sample of the bacteriophage L1 was added to the reaction tubes. After allowing time for colour development, the absorbances of the reaction tubes containing the standard amount of

DNA were compared to the tubes containing the bacteriophage L1 samples. The absorbance readings of the bacteriophage L1 samples were 0.062 for the 50 µl sample and 0.130 for the 100 µl sample which coresponded to DNA concentrations of ~300 µg/ml. The diphenylamine assay indicated that the purified samples of bacteriophage L1 contained DNA and the amount of DNA was equivalent to the amount of nucleic acid estimated by spectrophotometric analysis (Figure 11). The bacteriophage L1 was determined to be a DNA-containing bacteriophage.

### 3.11 Restriction enzyme mapping

#### 3.11.1 Restriction enzyme profile :

Forty-seven restriction enzymes were used to construct a restriction enzyme profile of the bacteriophage L1 genome (Table 6). The bacteriophage L1 genome, when run undigested, formed a single band which ran at a higher position than the largest fragment of the l *Hind* III standards which indicated that the DNA was most probably linear and greater than 23.1 kb in size. The size of the bacteriophage L1 genome was estimated using several restriction digests. The restriction enzyme *Sma* I routinely and clearly produced 10 single bands which when added together, showed the bacteriophage L1 genome to be approximately 50 kb in length. Other enzymes such as *BamH* I, and *Cla* I confirmed this finding (Table 7). Some restriction enzyme digests are shown in Plate 3.

#### 3.11.2, Restriction enzymes used for mapping.

The restriction enzymes useful for the restriction mapping of the bacteriophage L1 genome should ideally produce few (2 to 5) restriction fragments during a single digest and the fragments produced should be singlets (ie no two bands should be of the same molecular weight).

**Figure 11.** Burton Diphenylamine Assay Standard Curve. The standard curve generated for the Burton diphenylamine assay was used to determine whether the nucleic acid of bacteriophage L1 (measured by absorbance at 260 nm) was DNA or RNA. The diphenylamine assay showed that the estimated nucleic acid content of bacteriophage L1 sample as measured by absorbance (260 nm) was equivalent to the amount of DNA that was determined to be in bacteriophage L1 sample by the diphenylamine assay.

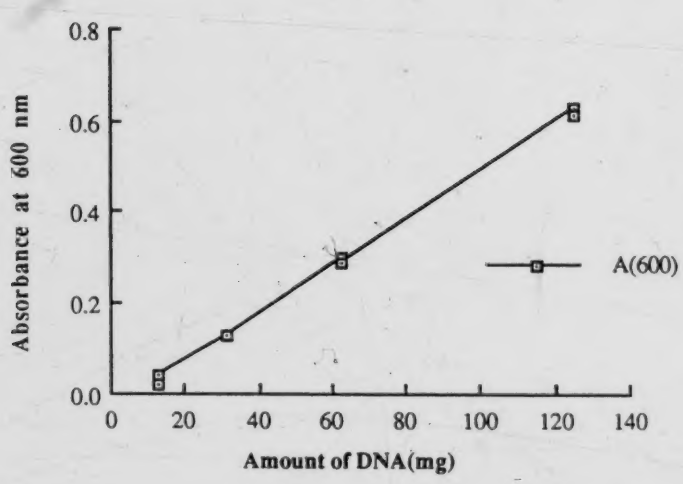

0.8
0.6
0.4
0.2
0.0
Absorbance at 600 nm
0
20
40
60
80
100
120
140
Amount of DNA(mg)
A(600)

**Table 6** : Restriction enzyme profile of bacteriophage L1

| Restriction Enzyme | No. of Bands | Restriction Enzyme | No. of bands | Restriction Enzyme | No. of bands |
|---|---|---|---|---|---|
| *Acc* I | 12 | *EcoR* V | 9 | *Pst* I | 9 |
| *Afl* II | 3 | *Hae* II | >20 | *Sac* I (*Sst* I) | 3 |
| *Alu* I | 1 | *Hha* I | >20 | *Sal* I | 1 |
| *Apa* I | 1 | *Hinc* II | 11 | *Sau96* I | 9 |
| *Ava* I | 13 | *Hind* III | 1 | *Sau3A* I | >20 |
| *Ava* II | 10 | *Hinf* I | >20 | *Sma* I (*Xma* I) | 10 |
| *Bal* I | 1 | *Hpa* I | 1 | *Sph* I | 1 |
| *BamH* I (*Bst* I) | 9 | *Hpa* II (*Msp* I) | >20 | *Sst* I (*Sac* I) | 3 |
| *Bcl* I | 12 | *Knp* I | 1 | *Sst* II | 12 |
| *Bgl* I | 10 | *Mlu* I | 4 | *Taq* I | >20 |
| *Bgl* II | 1 | *Msp* I (*Hpa* I) | >20 | *Tha* I | >20 |
| *Bssh* II | 1 | *Nar* I | 13 | *Xba* I | 3 |
| *Bst* I (*BamH* I) | 9 | *Not* I | 1 | *Xho* I | >20 |
| *Cla* I | 13 | *Nru* I | 12 | *Xma* I (*Sma* I) | 10 |
| *Dra* I | 1 | *Pvu* I | 1 | *Xor* II (*Pst* I) | 9 |
| *EcoR* I | 9 | *Pvu* II | 13 | | |

The enzyme in brackets is an isoschizomer which is defined as restriction endonuclease with the same recognition sequence but which may or may not produce identical cleavage products or ends (Bethesda Research Laboratories Catalogue and Reference Guide 1987).

**Table 7** : Analysis of the Size of Bacteriophage L1 Genome.

| Restriction Enzyme | Size (kb) | Total size(kb) |
|---|---|---|
| *BamH* I | 17.4, 8.7, 5.7, 5.2, 4.7, 2.4 | |
| | 2.2, 1.9, 1.5 | 49.7 |
| *EcoR* I | 10.7, 6.8(D), 5.7, 4.5, 3.8, | |
| | 3.2, 2.8, 2.6, 1.4 | 48.3 |
| *Pvu* II | 7.8 (D), 4.9, 4.1, 3.7, 3.5, | |
| | 3.3, 3.1, 3.0, 2.2, 1.9, 1.7, | |
| | 1.5, 1.1 | 49.6 |
| *Sma* I | 18.6, 11.7, 7.1, 5.3, 3.4, 1.5, | |
| | 1.1, 0.8, ~0.3, ~0.2 | 50.0 |

(D)= doublet band based on intensity of ethidium bromide staining

Plate 3. Restriction enzyme digests of Bacteriophage L1 DNA.

**3a.** Single restriction enzyme digests of bacteriophage L1 DNA which in part, produced the restriction enzyme profile and the estimate of the approximate size of the bacteriophage L1 genome. The restriction digested DNA was electrophoresesed on a 1.0% TB agaose gel.

**3b.** Single and double restriction enzyme digests of the bacteriophage L1 DNA used to approximate the size of bacteriophage L1 genome. The restriction digested DNA was electrophoresesed on a 1.0% TB agarose gel.

a
native DNA
λ std.
BamH I
Bgl II
EcoR I
Hind III
Kpn I
Pst I
Pvu I
Pvu II
Sal I
Sph I
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
b
Sst I
Sst I/Xba I
Xba I
Pst I/Sst I
Pst I
Pst I/Xba I
Sma I/Sst I
Sma I
Sma I/Xba I
λ std.
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-

From the restriction enzyme profile the following classes of restriction enzymes were distinguished :

| # of bands on the gel | Restriction enzyme |
|---|---|
| >10 | *Acc* I, *Ava* I, *Bcl* I, *Bst* I, *Cla* I, *Hae* II, *Hinc* II *Hinf* I, *Hha* I, *Hpa* II, *Msp* I, *Nar* I, *Nru* I, *Pvu* II, *Sau3A* I, *Sst* II, *Taq* I, *Tha* I, *Xho* I |
| 5-10 | *Ava* II, *BamH* I, *Bgl* I, *EcoR* I, *EcoR* V, *Pst* I, *Sau 96* I, *Sma* I, *Xma* I, *Xor* II |
| 2-4 | *Afl* II, *Mlu* I, *Sac* I, *Sst* I, *Xba* I |
| 1 | *Alu* I, *Apa* I, *Bal* I, *Bgl* II, *Bssh* II, *Dra* I, *Hind* III, *Hpa* I, *Kpn* I, *Not* I, *Pvu* I, *Sal* I, *Sph* I |

### 3.11.3 Single digests with *Sst* I and *Xba* I :

The most suitable enzymes for mapping were chosen from the restriction enzymes that produced from 2 to 4 bands. It should be noted that the number of bands produced by each restriction digest gave only a rough estimate of the number of restriction fragments produced as the bands may be composed of greater than one fragment. The enzymes *Sac* I and *Sst* I are isoenzymes and probably produced identical fragments. The enzyme chosen for mapping was *Sst* I, rather than *Sac* I because of the greater availability of *Sst* I in the laboratory. The enzymes *Afl* II and

*Mlu* I were less commonly available and fairly expensive so they were not used to construct the map. Thus, mapping was conducted using the restriction enzymes *Sst* I and *Xba* I. From the single digests of *Xba* I and *Sst* I (see Plate 3b), there appeared to be three restriction fragments formed by each of the digests as suggested by the three bands formed in each gel. However, the relative intensities of the bands and the failure of the bands to add up to 50 kb suggested that the bands seen in the gels were composed of more than 1 fragment each. The restriction fragments were of the following sizes : *Xba* I ~ 30, 8.5, and 6.6 kb *Sst* I ~ 13.8, 9, and 6.8 kb. In the *Xba* I digest, a doublet was suspected in the 6.6 kb band due to its more intense staining relative to the 6.8 kb *Sst* I fragment. Doublets were also suspected in the 13.8 kb and 9 kb *Sst* I bands also due to their intense staining (Plate 4).

### 3.11.4 Double digests with *Sst* I and *Xba* I :

The double digest of *Sst* I-*Xba* I produced apparently six bands when run on a wide-mini-sub™cell TA or TB agarose gel corresponding to the sizes of ~13.8, 8.5, 6.4, 3.9, 2.6, and 1.8 kb. When these fragment sizes were added up, the sum was still not 50 kb. A doublet was suspected in the 13.8 kb band. In order to resolve the 13.8 kb fragment, the restriction digests were run on a longer TA-gel (210 x 130 mm). The 13.8 kb fragment was actually composed of one 14.1 kb fragment and a 13.5 kb fragment (Plate 5).

### 3.11.5 Restriction enzyme map :

The large ~30 kb *Xba* I fragment was composed of the 13.5 and 14.1 kb *Sst* I fragments. Because the 13.5 and 14.1 kb *Sst* I fragments (13.8 doublet) were maintained in the *Sst* I-*Xba* I double digest, they could not contain any *Xba* I restriction sites. All the other *Xba* I fragments are less than or equal to 8.5 kb and could not have

**Plate 4**. Restriction enzyme digests of bacteriophage L1 DNA using the restriction enzymes *Sst* I and *Xba* I. Electrophoresis of the digested bacteriophage L1 DNA was conducted on a 1.0% TB gel using a wide mini-sub™ cell electrophoresis apparatus (Bio-Rad). The same banding pattern was produced by electrophoresis of the bacteriophage L1 DNA using a 0.6% TA gel electrophoresis (not shown).

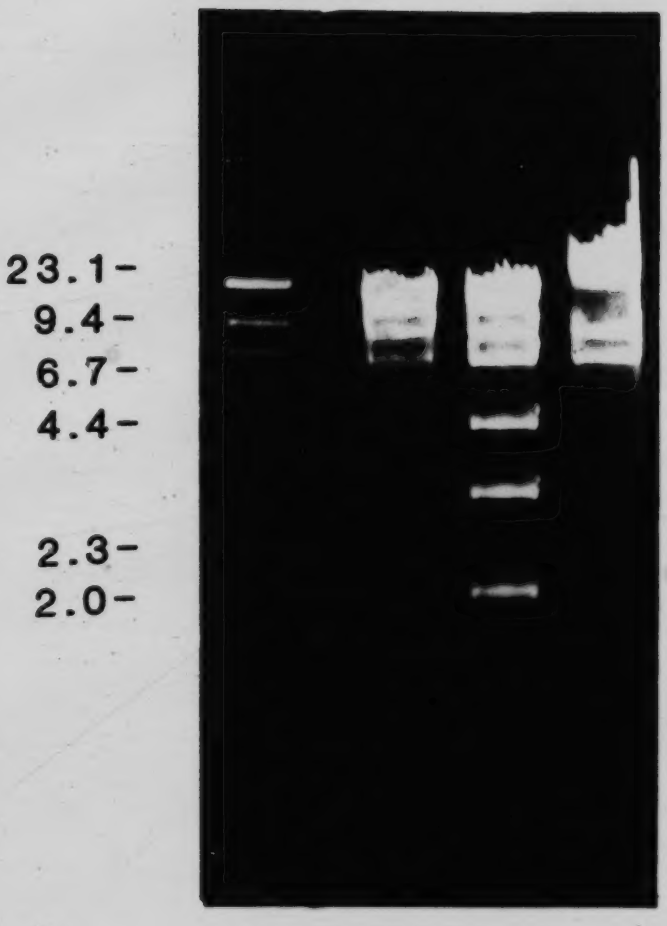
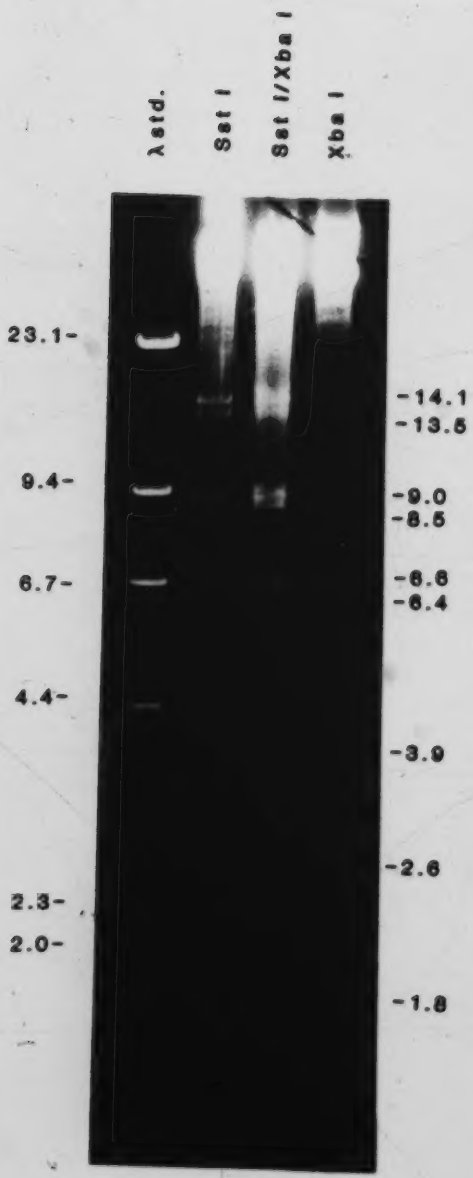

λ std.
Sst I
Sst I/Xba I
Xba I
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-

**Plate 5**. Resolution of the Large *Sst* I Restriction Fragments. Restriction enzyme digests of bacteriophage L1 DNAwith the enzymes *Sst* I and *Xba* I showing the actual number and intensity of the bands when a longer TA gel (210 x 130 mm gel apparatus) is run as compared with the wide mini-sub™ cell electrophoresis apparatus.


λstd.
Sst I
Sst I/Xba I
Xba I
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
-14.1
-13.5
-9.0
-8.5
-6.6
-6.4
-3.9
-2.6
-1.8

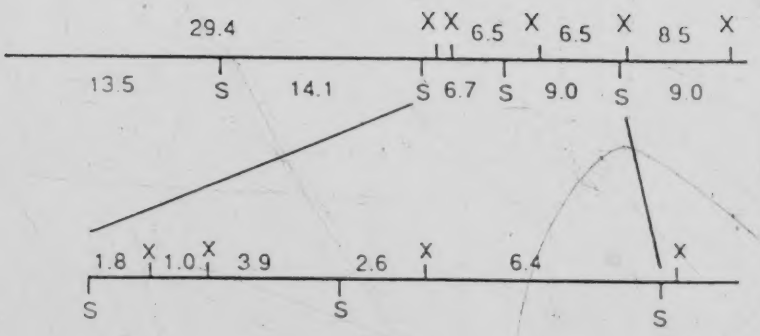
contained either the 13.5 or the 14.1 kb *Sst* I fragments. These results placed the 13.5 and 14.1 kb *Sst* I fragments in the 30 kb *Xba* I fragment. There was still a small amount of the 30 kb fragment not accounted for and either the 2.6 or the 1.8 kb fragment could possibly be included. To test this, the large 30 kb fragment was isolated from an agarose gel, purified using Geneclean™, redigested with *Sst* I, and run on a TB agarose gel. The resulting digest strongly showed the presence of the 1.8 kb fragment (Data not shown). There was also some evidence of fragment shearing through the manipulation of the 30 kb fragment which produced a ladder effect in the gel. However, the 1.8 kb fragment was very clearly more intensely stained than the sheared fragments leading to the conclusion that the 1.8 kb fragment was part of the 30 kb *Xba* I fragment. With the assignment of fragments to the large 30 kb *Xba* I fragment completed, the smaller *Sst* I and *Xba* I restriction fragments were studied. The 8.5 kb *Xba* I fragment must have been within one of the 9.0 *Sst* I fragments as it is maintained in the double digest. Again, the reasoning for this assignment was the same as that used for placing the 13.5 kb and 14.1 kb *Sst* I fragments within the 30 kb *Xba* I fragment. If the 8.5 kb *Xba* I fragment had an *Sst* I restriction site, it could not be maintained in the double digest. Since the 13.5 kb and 14.1 kb *Sst* I fragments are assigned to the 30 kb *Sst* I fragment, the only other *Sst* I fragment large enough to contain the 8.5 kb *Xba* I fragment was a 9.0 *Sst* I fragment. In support of this assignment of the 8.5 kb *Xba* I fragment to the 9.0 kb *Sst* I fragment is the fact that the 9.0 *Sst* I fragment was no longer present in the *Sst* I-*Xba* I double digest and the 8.5 kb fragment remained. One of the 6.5 kb *Xba* I fragments most probably was cleaved by *Sst* I digestion to a 3.9 and 2.6 kb fragments produced in the double digest. The sum of the 3.9 kb and 2.6 kb fragments is exactly 6.5 kb. The other 6.5 kb *Xba* I fragment appeared to be clipped to a 6.4 kb fragment by *Sst* I. This was evidenced by

the loss of the 6.5 kb *Xba* I fragment in the *Sst* I-*Xba* I double digest and the appearance of a 6.4 kb fragment. The *Sst* I fragments that were maintained in the *Sst* I-*Xba* I double digests were the 14.1 kb and 13.5 kb fragments. The doublet 9.0 kb *Sst* I fragments both had *Xba* I restriction sites, one being cleaved into an 8.5 kb fragment as mentioned above and the other most probably being cleaved into a 6.4 kb fragment and a 2.6 kb fragment. The 6.7 kb *Sst* I fragment posed a slight problem in that the *Sst* I-*Xba* I fragments that were remaining, the 1.8 kb and 3.9 kb fragments, did not add up to 6.7 kb. The assignment of the 3.9 kb and 1.8 kb fragments to the 6.7 kb *Sst* I fragment predicts the presence of a fragment of ~ 1 kb which should be present in the *Xba* I single digest and the *Xba* I - *Sst* I double digests. In an attempt to find the predicted 1 kb fragment, a 2% TB agarose gel and a 12.5% acrylamide gel were used to separate the restriction fragments from both a double digest (*Sst* I -*Xba* I) and a single *Sst* I digest. There was no 1 kb restriction fragment found in either digest. The restriction map of the bacteriophage L1 genome which was the most plausible is pictured in Figure 12.

### 3.11.6 End-labelling :

In order to determine which of the *Sst* I fragments and *Xba* I fragments were at the ends of the bacteriophage L1 genome, the DNA was subjected to an end-labelling reaction using T4 polynucleotide kinase. The enzyme T4 polynucleotide kinase was used because of its lack of specificity for both the γ-phosphate donor and the polynucleotide recipient and also the availability of the enzyme in the laboratory. The bacteriophage L1 genome could not be end labelled using T4 polynucleotide kinase indicating that the 5' end of the genome is not accessible to the enzyme. Lillehaug *et al.* (1976) reported the T4 polynucleotide kinase has preference for single-stranded 5'-

**Figure 12.** Restriction Map of Bacteriophage L1 Genome. The restriction map for the bacteriophage L1 genome has been constructed for the restriction enzymes *Sst* I (S) and *Xba* I (X). Map distances are given in kilobases (kb).


29.4
X X
6.5
X
6.5
X
8.5
X
13.5
S
14.1
S
6.7
S
9.0
S
9.0
1.8
X
1.0
X
3.9
2.6
X
6.4
X
S
S
S

hydroxyl termini, therefore, blunt-ended or recessed 5'-termini with 3'-overhangs would be poor substrates. In addition, single-strand nicks in the DNA are reported to also adversely affect the end-labelling reaction. From the results of the end-labelling experiment, the bacteriophage L1 genome could possess any of the three above mentioned features or alternatively the feature listed below (see section 3.11.7).

### 3.11.7 Reannealing experiment :

The bacteriophage L1 DNA was analysed for the possibility that it had its 5'-hydroxyl termini involved in a hairpin or a had protein covalently linked to its terminus. The reannealing experiment was done to compare the rate of reannealing of calf thymus DNA (which is double stranded, well characterized and has no unusual composition to its 5' end) to that of the bacteriophage L1 DNA. The results showed that the reannealing was comparable for the two DNA samples. The extracted bacteriophage L1 DNA appeared not to have a hairpin or a covalently bound protein at its 5' end.

## 3.12 Hybridization studies:

Hybridization studies were done in order to determine if bacteriophage homologous to the bacteriophage L1 were present in representative strains of the three *Bordetella* species that are mammalian pathogens. If the bacteriophage were not inducible, the only means of detection could be this probe. Any strains showing hybridization to the probe would be further studied to determine if the presence of the bacteriophage could influence the phenotypic properties of the organism. Also, the strains hybridizing with the bacteriophage could be grown under various conditions in an attempt to induce the bacteriophage.

### 3.12.1 Colony hybridization

Colony hybridization was used to screen the various strains of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* for the presence of DNA homologous to bacteriophage L1 DNA. The advantage of using this technique was that large numbers of colonies could be screened without having to purify DNA from the organisms. Disadvantages of the technique were that cellular debris may interfer with the results of the hybridization and that the amount of DNA present on the nitrocellulose filter was related to how quickly the various bacterial strains could grow. The resulting autoradiographs showed that the colonies of strains of *B. pertussis* and *B. parapertussis* tested did not hybridize with the bacteriophage L1 probe as well as the colonies of strains of *B. bronchiseptica* (Plate 6). Consequently, only the strains of *B. bronchiseptica* were analysed in the next hybridization experiments.

### 3.12.2 Dot-blot hybridization

Dot-blot hybridization was used to screen the strains of *B. bronchiseptica* that appeared to hybridize the L1 probe in the colony hybridization. This method used purified DNA which had the advantages of not having the cellular debris to interfer with the hybridization and that a measured standard amount of DNA was spotted onto the nitrocellulose filters. The strains showing hybridization to the bacteriophage L1 genome were as follows (Plate 7):

strong signals: AG1-L, AG9-L, AG11-L, AG11-H, 501, Tohama I bacteriophage lysate

weaker signals: Rab-10, BTS, Ft. Collins (Data not shown), Columbus

These bacterial DNA were used for the Southern blots.

**Plate 6.** Colony blot hybridization of the *Bordetella* spp., probing with bacteriophage L1 DNA. Strains from three species of *Bordetella* were grown on nitrocellulose filters, lysed and probed with nick-translated ($^{32}$P-dATP) bacteriophage L1 DNA. The photograph is of the autoradiograph of the blot of the following strains : (*B. bronchiseptica* =Bb, *B. parapertussis* = Bpp, *B. pertussis* = Bp)

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | blank | blank | blank | blank | Purified øL1 DNA | E. coli 364 |
| B | Bb Rat 1 | Bb Columbus | Bb 501 | Bb Ft. Collins | Bb Rab 10 | blank |
| C | Bb 214 | Bb 17640-SAC | Bb BTS | Bb AG1-L | Bb 87 | Bb 110-H |
| D | Bpp 77 | Bpp PL-1 | Bpp 17903 | blank | blank | blank |
| E | Bp 134 | Bp 3779+ | Bp Tohama I | Bp 18323 | Bp BB103 | blank |

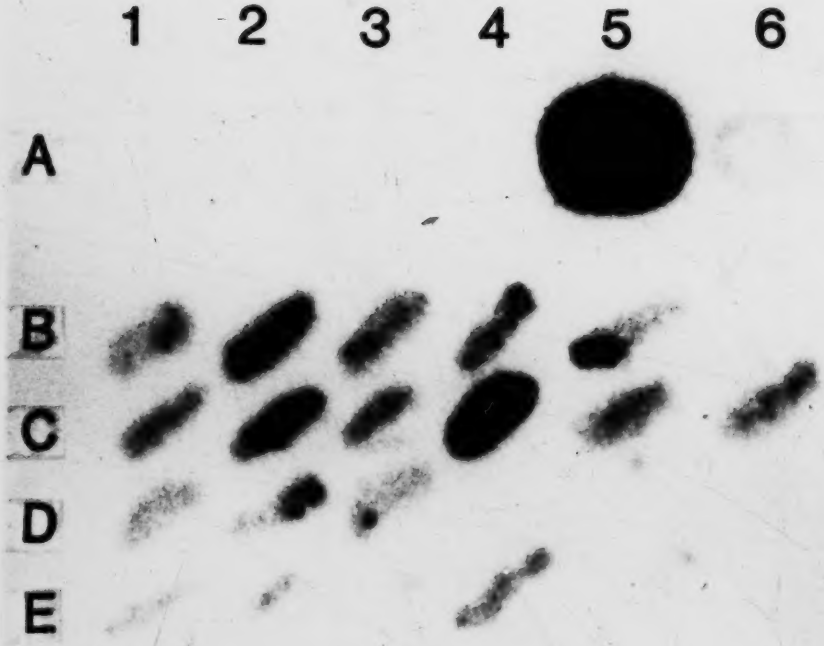
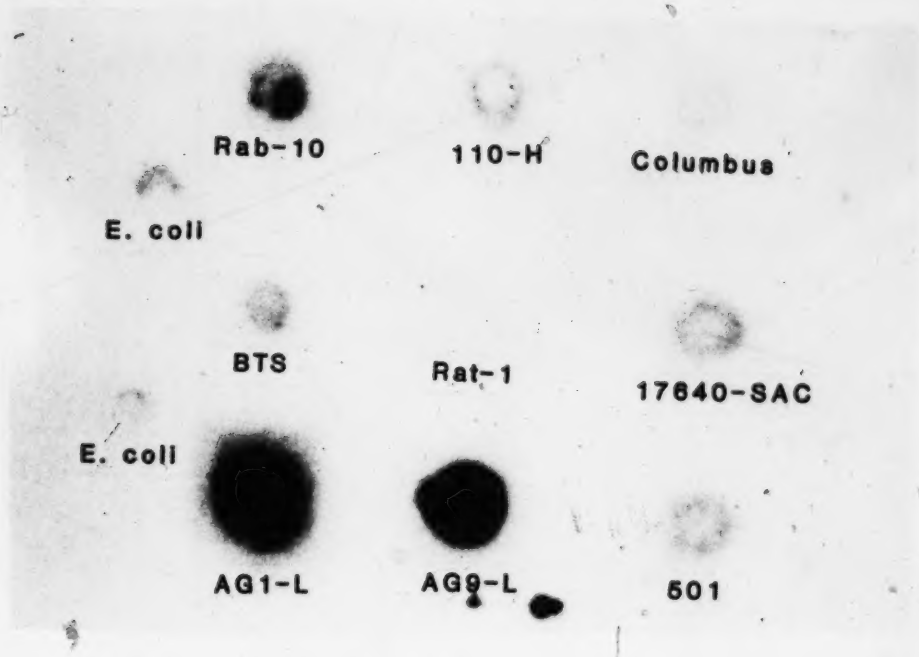
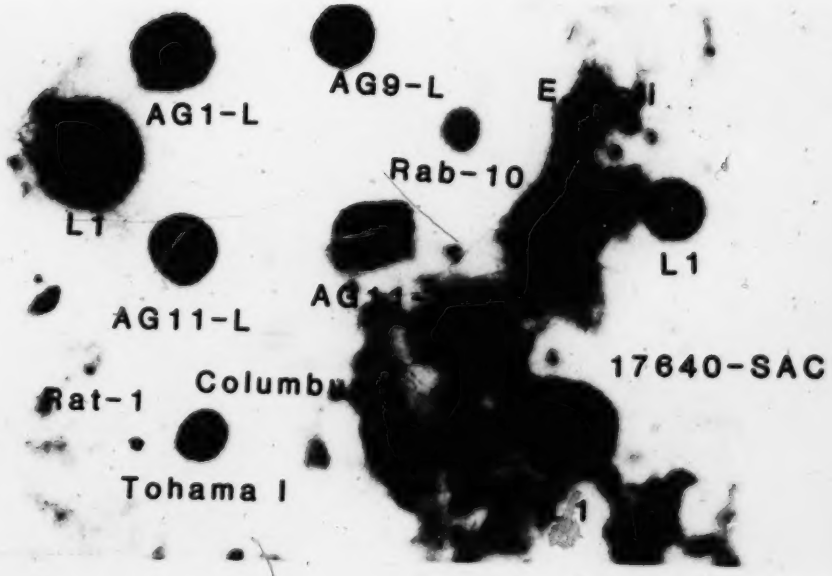

1
2
3
4
5
6
A
B
C
D
E

**Plate 7.** Two dot blot hybridizations of various strains of *B. bronchiseptica* with nick-translated bacteriophage L1 DNA. The dot blot hybridizations were conducted on two separate occasions, however, certain bacterial DNA samples were used in both blots. All strain names refer to strains of *B. bronchiseptica* with the following exceptions: E. coli = *Escherichia coli*; Tohama I = *B. pertussis* Tohama I bacteriophage crude lysate; L1 = purified bacteriophage L1 DNA)


Rab-10
110-H
Columbus
E. coli
BTS
Rat-1
17640-SAC
E. coli
AG1-L
AG9-L
501


AG1-L
AG9-L
Rab-10
L1
L1
AG11-L
17640-SAC
Rat-1
Tohama I

### 3.12.3 Southern-blot hybridization

The Southern blot hybridization was done to determine if the bacteriophage L1 was integrated into the bacterial chromosome of those strains of *B. bronchiseptica* that appeared to hybridize with the bacteriophage L1 genome by dot-blot hybridization. Any bacterial restriction fragments found to hybridize with the bacteriophage L1 DNA probe would be compared to the pattern produced by hybridization of bacteriophage L1 digested by the same restriction enzyme. Two possible patterns were predicted: 1) if the restriction patterns were the same between purified bacteriophage L1 DNA and the bacterial DNA, it would be assumed that the bacterial strain carried bacteriophage homologous to the bacteriophage L1 and that the bacteriophage genome was not integrated into the bacterial genome; 2) If the restriction pattern of purified bacteriophage L1 DNA was different than the bacterial DNA by only two fragments, this would indicate that the bacteriophage was incorporated into the bacterial genome with the ends of the bacteriophage genome integrated into the bacterial DNA.

The following strains of *B. bronchiseptica* showed hybridization to the bacteriophage L1 genome by Southern blot hybridization : AG1-L, AG9-L, AG11-H, and AG11-L (see Plate 8d). The restriction patterns produced by AG1-L, AG9-L, AG11-H, and AG11-L were identical to the restriction pattern produced by the purified bacteriophage L1 DNA using the restriction enzymes *BamH* I and *Xba* I (see Plate 8d). The bacteriophage L1 genome in these bacterial chromosomal digests was not integrated into the bacterial genome. The intensity of the bands and the relatively short exposure time (20 hours at -70°C) suggested a large copy number of the bacteriophage L1 in each of these samples. This suggested two possible explanations, one being that the bacteriophage L1 genome was carried as a multicopy plasmid within the host cells or, a more probable explanation was the bacterial culture had been induced to produce

**Plate 8** : Southern blot hybridizations of various strains of *B. bronchiseptica* with nick-translated bacteriophage L1 DNA. The concentration of DNA in the bacterial DNA samples was estimated by fluorometry (see section 2.19.2). Approximately 1 µg of the bacterial DNA was added to each restriction enzyme digest. One microgram of bacteriophage L1 DNA was also used in the restriction enzyme digests, however, a 1:100 dilution of the completed digest was also made with a small portion of the digest to be used as a positive control for the hybridization. After allowing the various restriction enzyme digestions to go to completion (~2 hours), the samples were run on a TB agarose gel and subjected to Southern transfer and hybridization. (Note that the undiluted bacteriophage L1 DNA sample produces the very strong hybridization signal whereas the diluted sample is not as overwhelmingly strong.)

**Plate 8a** : Ethidium bromide stained TB agarose gel of bacterial DNA samples treated with the restriction enzyme *BamH* I, which subsequently was subjected to Southern transfer and probed with nick-translated bacteriophage L1 DNA.

**Plate 8b** : Autoradiograph of the Southern blot of the agatose gel in plate 8a.

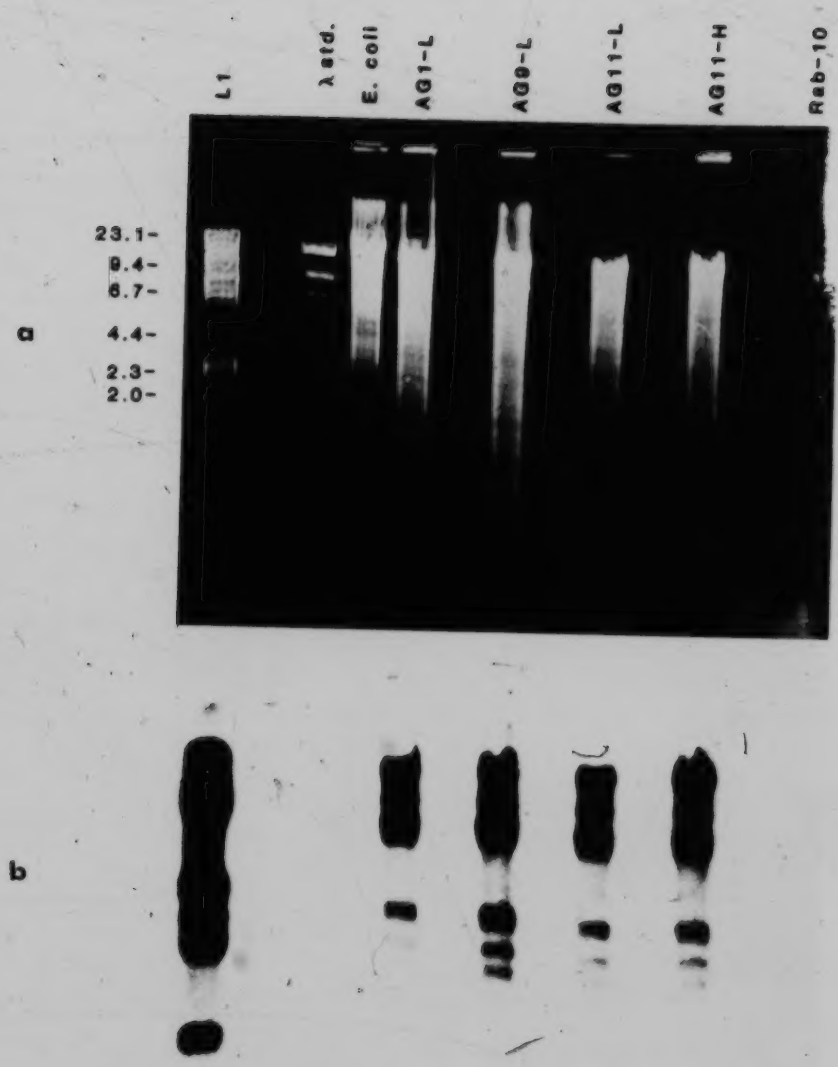

L1
λ std.
E. coli
AG1-L
AG9-L
AG11-L
AG11-H
Rab-10
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
a
b

**Plate 8c** : Ethidium bromide stained TB agarose gel of bacterial DNA samples treated with the restriction enzyme *BamH* I, which subsequently was subjected to Southern transfer and probed with nick-translated bacteriophage L1 DNA.

**Plate 8d** : Autoradiograph of the Southern blot of the agatose gel in plate 8c.

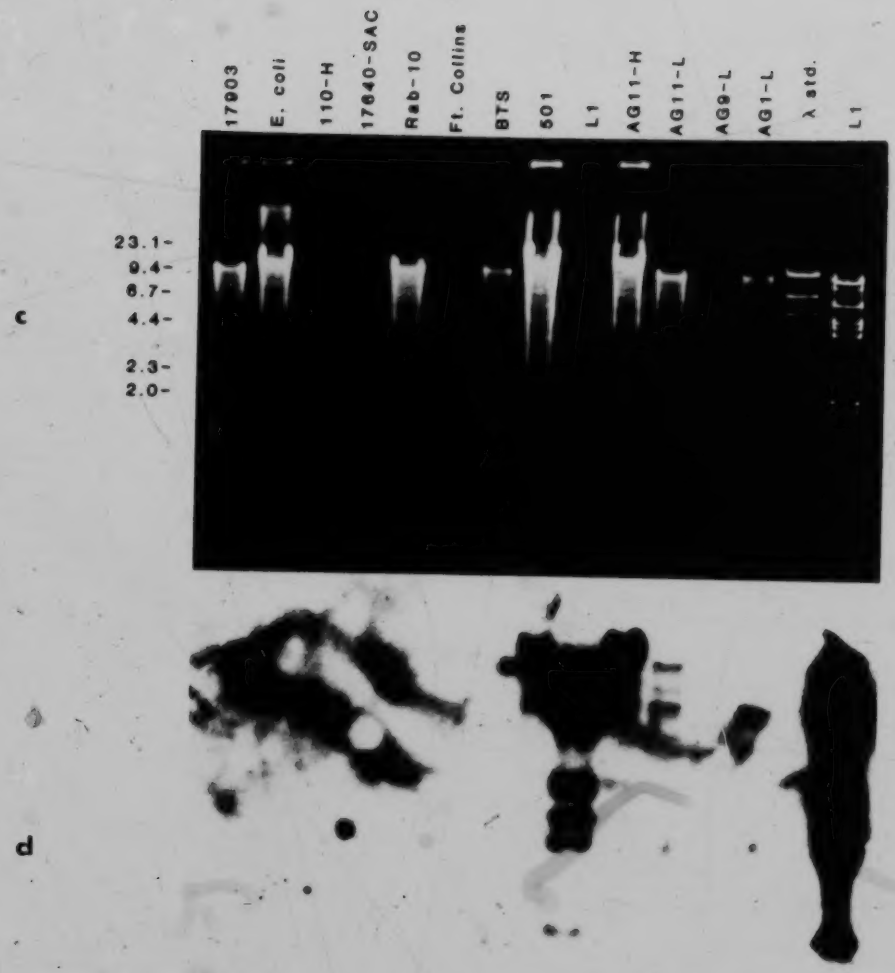
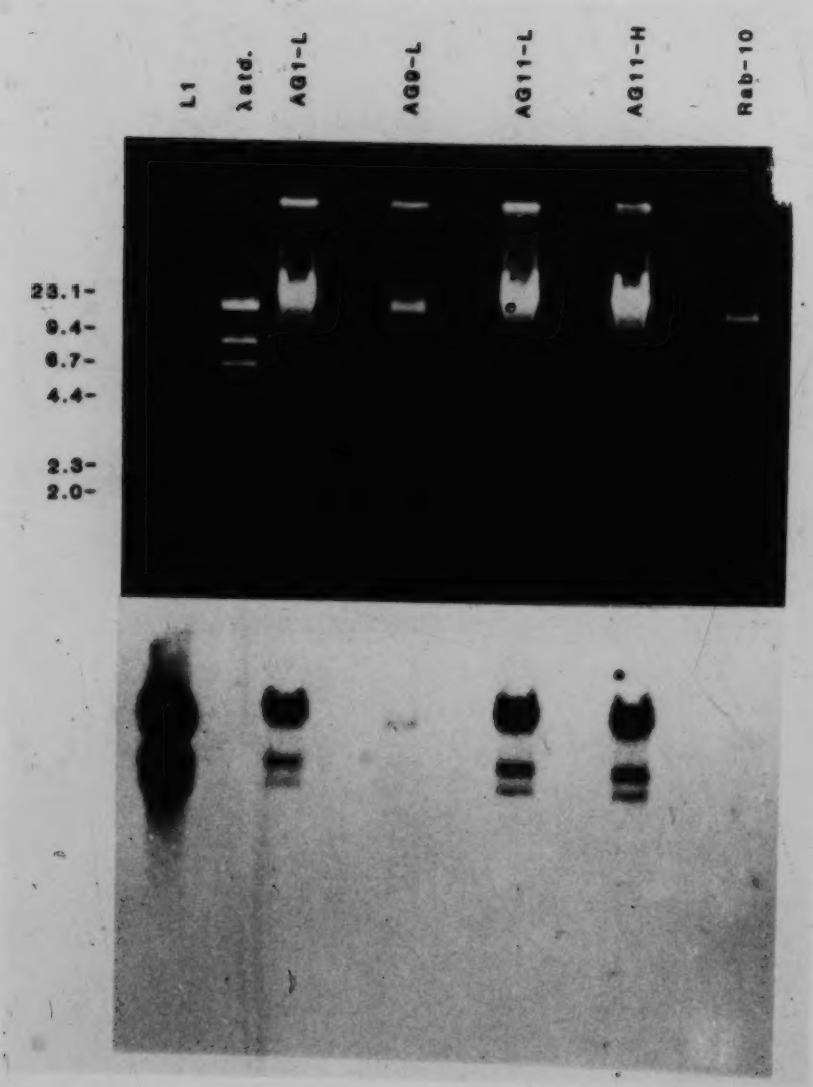

17903
E. coli
110-H
17640-SAC
Rab-10
Ft. Collins
BTS
501
L1
AG11-H
AG11-L
AG8-L
AG1-L
λ std.
L1
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
c
d

**Plate 8e** : Ethidium bromide stained TB agarose gel of bacterial DNA samples treated with the restriction enzyme *Xba* I, which subsequently was subjected to Southern transfer and probed with nick-translated bacteriophage L1 DNA.

**Plate 8f** : Autoradiograph of the Southern blot of the agatose gel in plate 8e.


L1
λstd.
AG1-L
AG9-L
AG11-L
AG11-H
Rab-10
23.1-
9.4-
6.7-
4.4-
2.3-
2.0-
e
f

bacteriophage during the procedure used to grow up the bacterial cells for DNA extraction.

A simple experiment was conducted to distinguish the two possible case scenarios. The strains of *B. bronchiseptica* in question, AG1-L, AG9-L, AG11-L, and AG11-H were inoculated into 100 ml of TSB and grown into stationary phase. One hundred microliter aliquots of broth cultures of the strains of *B. bronchiseptica* were removed at various time intervals and tested in a plaque assay for the presence of bacteriophage L1. The titer of bacteriophage in each flask after a 25 hour incubation time were the following : AG1-L $1 \times 10^8$, AG9-L $1 \times 10^5$, AG11-L $2 \times 10^8$, AG11-H $2 \times 10^5$ PFU/ml. This high titer of bacteriophage could account for the strong signal. It was not clear whether the bacteriophage L1 was carried as a lysogen, or if the bacteriophage L1 was a lytic bacteriophage in equilibrium with the bacterial host and not able to completely destroy the host cells *in vivo*.

One strain, *B. bronchiseptica* 501 weakly hybridized the bacteriophage L1 genome by the Southern blot hybridization technique (see Plate 8b). *B. bronchiseptica* 501 did not produce the same restriction pattern as the purified bacteriophage L1. This suggested the presence of DNA homologous to bacteriophage L1 DNA within the bacterial chromosome and the possibility of a bacteriophage in *B. bronchiseptica* 501. However, caution must be exercised in interpreting this result as the hybridizing band was found at the well of the agarose gel. Further Southern blot hybridizations should be conducted before considering *B. bronchiseptica* 501 as a lysogen for bacteriophage L1. The weaker signals from *B. bronchiseptica* strains Rab-10, BTS and Ft. Collins in the dot blot hybridizations (see Plate 7) did not produce restriction bands hybridizing more strongly than the negative controls in the Southern blots. Reasons for these negative results on the Southern blots include the possibility that even if the bacterial

strains carry bacteriophage, the bacteriophage may not be homologous to bacteriophage L1. All other reported *Bordetella* bacteriophage are noncontractile and may have poor homology to the bacteriophage L1 (Lapaeva *et al.* 1980). Insufficient amounts of bacterial DNA were applied to the gels making it less likely to obtain a strong hybridization signal perhaps explaining the discrepancy between results obtained from the dot blot and Southern blot hybridizations. Although, all bacterial DNA samples were equilibrated using fluorometry, there appeared to be RNA contamination in the DNA samples. The very strong hybridization of *B. bronchiseptica* strains producing bacteriophage L1 was a poor positive control for detecting a possibly lysogenic bacterial strain carrying a single copy of the bacteriophage genome in each bacterial cell.

# 4.0 Discussion :

## 4.1 Characterization of the Bacteriophage L1

The characterization of the bacteriophage L1 was conducted to fulfil the requirements for taxonomic classification of the bacteriophage as outlined by the International Committee for the Taxonomy of Viruses (I.C.T.V.) and as additional information for the characterization, a protein pattern and a restriction enzyme map were constructed.

### 4.1.1 Taxonomic Characterization (I.C.T.V. Guidelines)

The bacteriophage L1 can tentatively be placed within the I.C.T.V. taxonomic group, the family Myoviridae (Ackermann 1987) based on the morphology (section 4.1.1.2) and nucleic acid (section 4.1.1.1) of the bacteriophage L1.

#### 4.1.1.1 The Nucleic Acid

The nucleic acid was a single piece of linear double stranded DNA which was approximately 50 kb in length with no evidence of nucleotide base modification.

#### 4.1.1.2 The Virion Proper

The virion was morphologically described as a tailed, contractile bacteriophage with an icosahedral head, collar region, and distinctive base plate assembly with three or four spiked projections with knobby ends. The base plate assembly became clearly visible only upon contraction of the tail. The diameter of the bacteriophage L1 head was 56 nm in width (see section 3.5) which was comparable to the head measurements of bacteriophage λ but smaller than that of bacteriophage T4. Bacteriophage λ possesses an icosahedral head with a reported width of 54 nm (Kellenberger and Edgar 1970) whereas bacteriophage T4 possesses an anisometric head with a reported width of 85 nm (Earnshaw King and Eiserling 1978). The comparable head diameters of

bacteriophage L1 and bacteriophage λ appear to be reflected in the genome size of approximately 50 kb of double stranded DNA found in both bacteriophage. The bacteriophage T4 genome is also a double-stranded, linear DNA but is approximately 166 kb (Wood and Revel 1976). The bacteriophage T4 is approximately twice as big as the bacteriophage L1 and contains four times as much genetic material. Bacteriophage λ is approximately the same size as the bacteriophage L1.

The tail structures of the three bacteriophage are different. Bacteriophage λ possesses a long, flexible, non-contractile tail approximately 150 nm in length and ending in a single fiber. The bacteriophage T4 possesses a contractile tail like the bacteriophage L1, however, unlike bacteriophage L1, bacteriophage T4 has a hexagonal tail plate to which is anchored six long tail fibers and six shorter whiskers. This structure is very different from the bacteriophage L1 base plate structure.

The buoyant density of the bacteriophage L1 in cesium chloride was found to be approximately 1.5 g/ml, which is typical of most tailed bacteriophage (Ackermann 1974).

#### 4.1.1.3 Host Range of the Bacteriophage L1

The host range was found to be limited thus far to strains of *B. parapertussis* and *B. bronchiseptica*. The host range of the bacteriophage L1 is comparable to host ranges reported for other bacteriophage isolated from strains of *B. bronchiseptica* (Rauch and Pickett 1961). Rauch and Pickett (1961) reported that bacteriophage isolated from *B. bronchiseptica* were genus specific, that is forming plaques only on *Bordetella* species. In addition, these bacteriophage could not produce plaques on any strains of *B. pertussis* tested. It should be noted that bacteriophage L1 adsorbed to host bacterial cells optimally at lower temperatures (~4°C).

The bacteriophage L1 was found to be resistant to ether and chloroform but a severe drop in infectivity was noted on prolonged exposure to 1 M NaCl. Presumably, the bacteriophage particles were becoming disassociated on prolonged exposure to the high salt concentration as evidenced by observations of a thicker upper band of bacteriophage in the cesium chloride gradients if there was a delay (24 hours) in the purification procedure (see section 3.8). Purified bacteriophage L1 preparations were concentrated using 1 M NaCl, in addition to polyethylene glycol, to aid in precipitating the bacteriophage particles but the precipitated bacteriophage particles were placed onto the cesium chloride gradients without delay to minimize any deleterious effects of the salt. The bacteriophage L1 was also found to be stable if stored at 4°C in TSB for six months (time of observation) but loss of infectivity was noted at prolonged room temperature exposures.

Another criterion for bacteriophage classification recommended by the I.C.T.V. was investigation of the antigenic relationships between the bacteriophage. This was not done because the bacteriophage L1 was the only bacteriophage available from the *Bordetella* species at the time of the investigations. The bacteriophage isolated from *B. pertussis* Tohama I has been subsequently acquired and is a suitable candidate for future antigenic studies. From the preliminary dot blot hybridization study (see Plate 7), there is some DNA homology which may indicate some basis for an antigenic relationship between bacteriophage L1 and the Tohama I bacteriophage. In addition, the Southern blot hybridization revealed that a similar bacteriophage may be present in *B. bronchiseptica* 501. Further study of *B. bronchiseptica* strain 501 is warranted before firm conclusions can be drawn upon whether there is in fact, a bacteriophage within the *B. bronchiseptica* 501 strain and if the bacteriophage is inducible. Rauch and Pickett (1961) reported the inability to induce the bacteriophage of *B.*

*bronchiseptica* strains with ultra-violet light. However, growing the bacterial strains AG1-L, AG9-L, AG11-L and AG11-H to stationary phase either induced bacteriophage L1 or caused an accumulation of bacteriophage L1, if it was in fact being spontaneously produced throughout the growth cycle of the bacteria. The bacteriophage possibly present in *B. bronchiseptica* 501 was not inducible by growth of the bacterial host cells to stationary phase. Alternative means such as mitomycin-c induction or growth of the organisms in different media to stress the organisms, causing the induction the bacteriophage may have to be employed.

### 4.1.2 Properties of Bacteriophage L1 not required for Taxonomic Classification of Bacteriophage

As of yet, there is no further taxonomic subdivision of the family Myoviridae, the family of contractile-tailed bacteriophage into which the bacteriophage L1 has tentatively been placed. Tailed bacteriophage are the largest viral group known, with approximately 2721 reported observations of tailed bacteriophage, of which contractile tailed bacteriophage comprise 811 of the observations (Ackermann 1987). However, within the genus *Bordetella*, the bacteriophage L1 is the only reported contractile tailed bacteriophage to date (personal communications Dr. H.-W. Ackermann). Because of the bewildering numbers of tailed bacteriophage, the need to further characterize bacteriophage L1 to identify unique properties of the bacteriophage became more apparent.

#### 4.1.2.1 Protein Pattern

The bacteriophage L1 produced a protein pattern different than those produced by either bacteriophage T4 or bacteriophage λ, differring not only in the relative numbers of proteins but also in the molecular weights of the major protein bands. The molecular weights of the major protein bands produced by bacteriophage T4 and

bacteriophage λ compared favourably to the published values for the molecular weights of major head proteins of the two bacteriophage (see section 3.10). The major proteins of the bacteriophage L1 could not be stained with the silver staining technique. In addition, the bacteriophage L1 protein bands present in the silver stain were also present in the autoradiographs of the $^{125}I$ surface-labelled bacteriophage. This suggests that all proteins visualized in the silver stain are proteins with an exterior exposure. The silver stained bacteriophage proteins could possibly be surface labelled due to the bacteriophage L1 particles being lysed during the iodination procedure. However, if this were the case, the bacteriophage L1 DNA would also be labelled and one would expect to see a smear over the surface-labelled proteins. In fact, during one of the surface labelling experiments, a smear was observed that obscured the protein bands. The reason for the lysed bacteriophage particles in that particular experiment was unknown. All subsequent iodination experiments were clear of this smear. Because all detected proteins are external proteins, there was no evidence for the presence of internal enzymes. For a more detailed analysis, the bacteriophage L1 would have to be intrinsically labelled with either $^{14}C$ or $^{35}S$-methionine.

The number of bands produced in each protein profile of bacteriophage T4, bacteriophage L1, and bacteriophage λ should correspond with the amount of DNA possessed by each bacteriophage. However, the proteins coded for by the bacteriophage genome may not all be structural proteins present in the final bacteriophage particle but may be necessary in assembly of the bacteriophage or be modified by cleavage from a larger precursor protein to form the structural proteins. A crude estimation of the number of structural proteins expected in bacteriophage L1 as a function of its genome size can be made however, from reported findings in the literature correlating the of number of structural proteins with the amount of nucleic acid

and structural complexity of other well-characterized bacteriophage. Estimations of the number of proteins coded for by bacteriophage T4 and bacteriophage λ genomes were made from the following approximations :

> "The T4 genome is composed of nucleotide triplets made up of $2 \times 10^5$ base pairs which probably encodes $2 \times 10^5 / (3 \times 330) = 200$ different proteins having an average of 330 amino acids in their chains. It would seem most likely that much less than half of these 200 proteins are component parts of the infective T-even phage, with only about forty different protein species having been identified in the phage particle."
> (from Stent and Calendar (eds.) 1978)

Bacteriophage λ has sufficient DNA to encode approximately 50 proteins of molecular weight 33 kD, with approximately 35 genes known (Hendrix 1970).

There are at least 18 different genes concerned with bacteriophage λ morphogenesis. Seven genes control the synthesis of the phage head and DNA maturation. The remaining 11 genes control the synthesis of the tail proteins. Only eight subunits of different electrophoretic mobilities are observed in the extracts of the particles. It is suspected that additional proteins should be present (Murialdo and Siminovitch 1971).

Of interest with regards to structural complexity coupled with paucity of genetic material is the *Bacillus subtillis* phage ø29, which contains 18 genes but is a complex contractile, tailed phage.

Since the size of the bacteriophage L1 genome (~50kb) is comparable to the bacteriophage λ, a comparable number of proteins of molecular weight 33kD should be coded for by the bacteriophage L1 genome (~50 proteins). In addition, due to the structural complexity of the bacteriophage L1 contractile tail, it might be estimated that greater than the 18 different genes required for bacteriophage λ morphogenesis would be required for the construction of the bacteriophage L1. However, the example of the *B. subtilus* bacteriophage ø 29 indicates that fewer than 18 genes are necessary for the contractile tailed bacteriophage morphogenisis. Experimentation would be the best way to determine which genes are essential for morphogenesis. This information would be vital for the construction of cloning vectors (see section 4.4).

#### 4.1.2.2 Restriction Enzyme Analysis

Restriction enzyme analysis showed that bacteriophage L1 does not appear to have DNA modified by either methylation, as the hydroxy-methyl cytosine modification of bacteriophage T4 DNA or by glycosylation. It is unlikely that the DNA of bacteriophage L1 is methylated since the restriction enzymes *Ava* I, *Ava* II, *Pst* I, *Pvu* II, *EcoR* I, and *Taq* I, which are unable to cleave methylated DNA, cleaved bacteriophage L1 DNA (BRL Catalogue and Reference Guide). The bacteriophage L1 DNA is resistant to cleavage by the restriction enzyme *Hind* III. This possible modification to the *Hind* III restriction site is consistant with results of two published reports on *Bordetella* DNA. Greenaway (1980) reported a *Hind* III-like restriction activity from a derivative of *Bordetella pertussis*, however, modification to the *Hind* III sites was not shown. Weiss and Falkow (1982) demonstrated that DNA (plasmids) isolated from *Escherichia coli* could not be introduced into *B. pertussis* if it contained *Hind* III restriction sites and that *Hind* III sites are modified by *B. pertussis*.

### 4.1.2.3 Analysis of the Termini of the Bacteriophage L1 DNA.

The reannealing experiment (see section 3.13.5) showed no evidence to suggest that bacteriophage L1 DNA had a hairpin structure, or a covalently bound protein or a nick at the 5'-hydroxyl termini of the genome. However, the T4 polynucleotide kinase was not able to incorporate the γ-phosphate donor into the 5'-hydroxyl termini. A possible cause for this was bacteriophage L1 may possess a recessed 5'-termini with 3'-overhangs or blunt ends (Lillehaug *et al.* 1976). The Bethesda Research Laboratory Catalogue and Reference Guide (1987) stated that poor kinase effeciency may be due to self-annealing of G-C rich ends. The mol % G + C of the DNA of the mammalian respiratory species of the genus *Bordetella* is 66-70 ($T_M$) (Pittman 1984). Speculation as to the nature of the ends of bacteriophage L1 DNA and the fact that no incorporation of the γ-phosphate donor suggested the possibility of G-C rich sticky ends with a recessed 5'- termini with a 3'-overhang. A formal experiment to detect the presence of sticky ends was not done, but, none of the restriction digests produced any difference in banding pattern, regardless of whether the digest was heated to 65°C prior to running it on the gel or just loaded onto the gel after digestion at room temperature or 37°C (depending on the enzyme). The $T_M$ for cohesive termini generated by restriction enzymes (4 bases) is around 5°C (Kaiser and Murray 1984). If there was a short G-C rich 3'-overhang, it wouldn't be detected by running the DNA digests at 37°C as compared with 65°C. A simple way to determine if there are sticky ends at the bacteriophage L1 genome termini would be to heat the purified bacteriophage L1 DNA to 65°C for 10 minutes followed by rapid cooling on ice and running the DNA samples on an agarose gels. Since circularized DNA migrates differently than a linear DNA of the same molecular weight in the agarose gel during electrophoresis, a distinction could

be made between DNA that had been circularized by the heating and rapid cooling and a control DNA that had not been heated and rapidly cooled.

**4.2 Relationship of Bacteriophage L1 to the Bacterial Host .**

Bacteriophage, like all viruses, are metabolically inert and depend solely on host cells for propagation either by the lytic or lysogenic cycle. The bacteriophage L1 was isolated from *B. bronchiseptica* AG1-L which spontaneously released bacteriophage L1 in broth culture of the bacteria. The bacteriophage L1 was present in three other monkey isolates of *B. bronchiseptica* from the same case of bordetellosis as determined by the colony blot, dot blot and Southern blot hybridization (see Plates 6, 7and 8). From the Southern blots, it was evident that the unintegrated bacteriophage DNA was in such great quantities as to obliterate any evidence of bacteriophage DNA integration into the bacterial chromosome. It was not possible to tell whether or not the bacteriophage L1 was integrated into the bacterial chromosome. Large amounts of bacteriophage DNA corresponding to significant titers of bacteriophage L1, were isolated from broth cultures of the monkey isolates that were in stationary (perhaps death) phase. This suggested that if bacteriophage L1 was a temperate bacteriophage conferring a lysogenic phenotype to the host bacterial cells, the bacteriophage may have been induced to enter the lytic cycle at this advanced stage of bacterial growth. It should be noted that even at 12-14 hours of incubation on the nitrocellulose filters for the colony blot, the hybridization between the labelled bacteriophage L1 DNA and the DNA of the colonies of *B. bronchiseptica* (monkey isolates) were very strong. In order to detect bacteriophage L1 integrated into the bacterial chromosome, assuming that it is integrated in the first place, the parental *B. bronchiseptica* strains should be harvested in a very early stage of their growth phase. Alternatively, bacteriophage L1 may be a pseudolysogen for *B. bronchiseptica* strains but lytic for *B. pararpertussis* strains as

evidenced by the carriage of the bacteriophage L1 even in clinical isolates of *B. bronchiseptica*. The ability of bacteriophage L1 to form plaques on other strains of *B. bronchiseptica* may exhibit the selective advantage that the *B. bronchiseptica* AG1-L, AG9-L, AG11-L, and AG11-H have in being able to survive the infection of a lytic bacteriophage. Resistance to bacteriophage infection may also confer changed external bacterial cell structures such as surface antigens and virulence determinants. Modification of bacterial cell surface is one way in which pseudo-lysogeny can be established. Perhaps other strains of *B. bronchiseptica*, not having the modified receptors for the bacteriophage L1 are selected against. Enhanced virulence may be selected for in the bacterium due to the selective pressures of the lytic bacteriophage. However, carriage as a commensal organism as seen in several of the reported isolations of *B. bronchiseptica* (Little 1975, Giles *et al.* 1980, Rutter 1981) would appear to be the ideal lifestyle for a pathogenic bacterial species. It would seem counterproductive for the bacterial species to increase its virulence when it appears to be establishing a more commensal lifestyle with its mammalian hosts. The role of the bacteriophage in the *Bordetella* species should be more carefully explored.

The question of whether the bacteriophage is carried as a pseudolysogen or a true lysogen may serve to illuminate the mechanism of the alterations in the phenotype of bacterial pathogens which may occur under the influence of bacteriophage. Recent studies of the bacteriophage of *Vibrio cholerae* indicate that lysogeny may not be essential for the increased virulence of strains with bacteriophage associated with them. There is a striking similarity between *Bordetella* bacteriophage and interaction with their host cells and the bacteriophage of *V. cholerae* and their host cells.

"*Vibrio chlolerae* is divided into two biotypes, classical and El Tor. Workers have demonstrated a possible relationship between lysogeny and virulence of

the El Tor strains. El Tor strains could be typed into two groups, those that release temperate phage and those who do not. Upon examination of the El Tor strains that release phage appeared to be the cause of a more severe disease while the non-releasers were associated with mild or relatively asymptomatic cholera. Phage conversion has been reported in the form of changes in serotype. The isolates surviving phage infection produced altered agglutination reactions and unlike those of the classical biotype, were variable and highly unstable, with large proportion agglutinating with anti-rough antisera. Strains maintaining their converted characteristics were resistant to infection by CP-T1 (putative transducing phage) but could not be induced to release bacteriophage therefore it is not known if the stable seroconverted strains were lysogens. Phage conversion by CP-T1 of the two cholera biotypes is markedly different. The variation in surface antigens on phage treated El Tor strains may be due to selection of phage resistant derivatives, which have various degrees of roughness and therefore different reactivity with the antisera. In contrast , classical strains appear to undergo a more defined conversion, which may be related directly to lysogeny by CP-T1. These phenomena are complicated by the fact that CP-T1 uses the O antigen as its receptor. Thus, phage treatment may simply be selecting resistant mutants and not convertants. The temperate nature of CP-T1 is in doubt and probably produces pseudolysogens. Guidolin and Manning also were unable to demonstrate lysogeny by CP-T1. By Southern hybridizations with cloned CP-T1 DNA fragments, they were unable to detect CP-T1-related sequences in *V. chlorae* strain 1633, a supposed CP-T1 lysogen, even though this strain had all the other characteristics of *V. chlorae* strain 1633." (Guildoloin and Manning 1987)

This is very similar to the finding of Paloheimo *et al.* (1987) and Mebel *et al.* (1980) with the pertussis bacteriophage. Paloheimo *et al.* (1987) reported not being able to detect the Tohama I bacteriophage in the parental strain of *B. pertussis* Tohama I. Bacteriophage L1 was found in certain strains of *B. bronchiseptica*, however not in the integrated form expected of a true lysogen but rather as the virulent form of bacteriophage, that is in its lytic cycle with several copies of the bacteriophage L1 having been produced. Whether the bacteriophage L1 is a pseudolysogen selecting for virulent organisms *in vivo* , or a true lysogen encoding information that converts the host cells to a more virulent form, may have the same effect for susceptible mammalian hosts.

**4.3 Contribution of Bacteriophage to the Virulence of the *Bordetella* species.**

The possible influence of bacteriophage on the virulence of the *Bordetella* species has been a recent avenue of research. Bacteriophage isolated from several strains of *B. bronchiseptica* (Rauch and Pickett 1961), and more recently, strains of *B. pertussis* (Mebel *et al.* 1980) have been reported. The presence of bacteriophage in any bacterial pathogen warrants investigation since bacteriophage are known, in certain cases, to alter phenotypic properties including the virulence of susceptible bacterial hosts (see section 1.5.2.1). The possibility of a role for bacteriophage in the virulence of the *Bordetella* species, particularly the possibility that bacteriophage L1 may contribute to the virulence of *B. bronchiseptica* , will be discussed in the context of the present knowledge of both the known virulence determinants of the *Bordetella* species and also the reported influences of bacteriophage on the *Bordetella* species.

### 4.3.1 Virulence Changes Influenced by Bacteriophage of *B. pertussis*

The bacteriophage isolated from *B. pertussis* Tohama I was reported to increase the virulence and alter certain phenotypic characteristics of heterologous strains of *B. pertussis* which were judged to be newly lysogenic for the bacteriophage Tohama I (Mebel *et. al.* 1980) (see section 1.4.1). Further, Mebel and Lapaeva (1982) reported the conversion of the serotype of *B. parapertussis* strains to serotypes related to those of *B. pertussis* under the influence of the bacteriophage Tohama I. A proposal was forwarded that the bacteriophage Tohama I was a transducing bacteriophage, encoding regulatory genes for the expression or enhancement of the virulence of *B. pertussis* strains (Mebel *et al.* 1980). The reported conversion of *B. parapertussis* to *B. pertussis*-like organisms under the influence of the putative transducing bacteriophage Tohama I fuelled speculation that perhaps *B. pertussis* isolates were lysogenic strains of *B. parapertussis* (Granstrom and Askelof 1982). However, other workers do not support the hypothesis of a transducing bacteriophage producing virulent *B. pertussis* strains from the less virulent *B. parapertussis* strains (Musser *et al.* 1986, Arico and Rappuoli 1987, Paloheimo *et al.* 1987) (see section 1.4.1). Although the reports of bacteriophage involvement in changes in serotype, antibiotic sensitivities, virulence and other phenotypic characteristics of *B. pertussis* have not been reproduced, the original observations of Mebel *et al.* cannot be totally discounted. The possible effects of changing the antigenic nature of the *Bordetella* species under the influence of bacteriophage could have major ramifications on the design of vaccines against pertussis in humans or bordetellosis in animals to include antigens encoded by bacteriophage, perhaps in the form of antibodies to purified bacteriophage. The bacteriophage L1, being isolated from a virulent strain of *B. bronchiseptica*, could possibly influence the virulence of this strain of *B. bronchiseptica* perhaps by encoding

genes whose products enhance or express virulence determinants for *B. bronchiseptica* or possibly alter the antigenic nature of this strain in such a way to make it more resistant to the animals immune system.

### 4.3.2 Pathogenicity of *B. bronchiseptica.*

*Bordetella bronchiseptica* has been recognized as a mammalian respiratory pathogen since 1910 but the mechanism(s) by which the organisms cause their pathogenic effect are still unresolved (Ferry 1910, Goodnow 1980). A complicating factor in the understanding of the pathogenesis of the disease is the reported variation in virulence of the *B. bronchiseptica* isolates. Much of the current research into the pathogenesis of *B. bronchiseptica* is being conducted in the swine industry where there is considerable economic losses due to infectious atrophic rhinitis (Goodnow 1980 Roop 1987). Several researchers have reported variations in the pathogenicities of *B. bronchiseptica* isolates for pigs. Ross *et al.* (1967) reported intranasal inoculations of pigs with strains of *B. bronchiseptica* isolated from swine, rabbits, cats, and rats caused only mild to moderate turbinate atrophy. The same experiment showed that an isolate of dog origin caused no turbinate hyperplasia or turbinate atrophy. Skelley *et al.* (1980) and Miniats and Johnson (1980) reported that several *B. bronchiseptica* isolates from hogs produced differences in the severity of infection in other susceptible swine. Skelley *et al.* also found that only 50% of the porcine isolates produced any form of nasal turbinate atrophy in the inoculated swine. In addition, several reports of the presence of *B. bronchiseptica* in the respiratory tracts of swine considered to be free from clinical respiratory disease indicate that the colonization of the host may not necessarily produce disease (Little 1975 Giles *et al.* 1980 Rutter 1981). Collings and Rutter (1985) concluded that development of turbinate atrophy was associated with the ability to produce heavy, persistent colonization of the nasal cavity coupled with the

production of heat-labile or dermonecrotic toxin. Roop *et al.* (1987) also reported considerable variation among strains of *B. bronchiseptica* in their ability to infect and produce lesions in young pigs but found a positive correlation between virulence and the quantitative production of dermonecrotic toxin . In addition, Roop *et al.* (1987) determined that some phenotypic characteristics that had previously been associated with virulent or phase I organisms, namely domed, smooth colony surface, hemolytic ($Dom^+$ $Scs^+$ $Hly^+$) colonial phenotype on Bordet-Gengou agar, hemagglutination, *in vitro* attachment to ciliated epithelial cells, and adenylate cyclase activity, did not always correlate with *in vivo* virulence for the *B. bronchiseptica* strains examined (see section 1.2).

That bacteriophage may be influencing the virulence of the *B. bronchiseptica* isolates becomes a possibilty in light of the unknown nature of the virulence of *B. bronchiseptica* isolates, the possible influence of bacteriophage Tohama I on *B. pertussis* and *B. parapertussis* strains, and the reported isolation of bacteriophage from thirty eight of forty eight strains of *B. bronchiseptica* isolated from several mammalian hosts (Rauch and Pickett 1961). The strain of *B. bronchiseptica* from which bacteriophage L1 was isolated came from an especially virulent outbreak of bordetellosis in a Green monkey colony. *B. bronchiseptica* has been associated with pneumonia in primates. Seibold *et al.* (1970) reported that 27% of bronchopneumonia cases in *Calicebus* species primates were associated with *B. bronchiseptica*. The bacteriophage L1 may be contributing to the virulence of this particular strain of *B. bronchiseptica*. The bacteriophage L1 may or may not contribute to the virulence of other strains *B. bronchiseptica* isolated from mammalian host other than primates. This may partially explain the host specificity of *B. bronchiseptica* isolates. Bacteriophage may influence the surface components of the bacterial cells which may in turn,

determine which mammalian host is susceptible for colonization and infection both within species and genera of mammals. Further investigation of this hypothesis may include a comparison of various mammalian hosts infected with various *B. bronchiseptica* strains either carrying the bacteriophage L1 or cured of the bacteriophage L1. Examination of the colonization, virulence and expression of putative virulence factors would be possible. Barring the use of animals, assaying various strains of *B. bronchiseptica* for virulence determinants upon exposure to the bacteriophage L1 could be useful in illuminating the possible effect of the bacteriophage L1 on the virulence of *B. bronchiseptica* isolates.

### 4.3.3 *B. parapertussis* and *B. avium*

*B. avium* is a relatively new discovery therefore even less is known about organism with respect to pathogenesis than the other members of the *Bordetella* species. The organisms are serologically related to *B. bronchiseptica* strains isolated from swine (Hinz *et al.* 1979). They also have a similar epidemiology, with young birds being infected more often than older birds, showing similar histopathology and clinical symptoms to *B. bronchiseptica* swine isolates. If the bacteriophage L1 can plaque on strains of *B. avium*, this can be seen as further evidence that *B. avium* belongs in the genus *Bordetellae*. In addition, examining the virulence of the *B. avium* isolates in relation to the bacteriophage L1, similar to the way proposed for the *B. bronchiseptica* isolates may elucidate the pathogenesis of the *B. avium* for the young birds.

No bacteriophage have been reported to date from strains of *B. parapertussis*, however *B. parapertussis* strains appear to be host cells to both bacteriophage isolated

from *B. pertussis* and *B. bronchiseptica*. The role of bacteriophage in the pathogenesis of *B. parapertussis* infections is unknown.

### 4.4 Relevance of Bacteriophage L1

As stated earlier in the discussion, the bacteriophage L1 can not be discounted from adding to the virulence of the *B. bronchiseptica* strains. 1) There is evidence that *B. pertussis* strains have an altered virulence phenotype influenced by their bacteriophage; 2) Bacteriophage have been isolated from several *B. bronchiseptica* strains, and there is a great variability of virulence among *B. bronchiseptica* strains, even within the same species of mammalian hosts, which prevents attempts to correlate known virulence factors with all cases of the disease. Bacteriophage influence on the virulence of *B. bronchiseptica* should be examined more closely.

A practical extension of the present work is that bacteriophage L1 could be developed as a cloning vector. An advantage would be the high copy number of the bacteriophage produced as compared to that achieved by plasmids. The bacteriophage L1 could be propagated on several strains of *B. parapertussis* and *B. bronchiseptica* as suggested by the ability of the bacteriophage L1 to plaque on the above mentioned hosts. In addition, the recombinant bacteriophage will infect viable, susceptible host cells without having to make the bacterial cells competant as is necesary for transformation experiments. The bacteriophage DNA appears to contain the same modifications as the host *Bordetella* species (see section 4.3.3) such that the cloning vector DNA will not be degraded by the host restriction-modification system. The bacteriophage L1 is also very stable, and can be stored at 4°C for as long as six month periods.

To create a cloning vector, the genome of the bacteriophage L1 must be mapped in order to determine which genes are essential for bacteriophage propagation and those which are not. After introduction of foreign DNA into the cloning vector, the recombinant DNA must be of a suitable length to package once it has been transfected into a suitable host cell. Plaques cannot form if the recombinant bacteriophage DNA does not have the necessary information to code for a bacteriophage particle. Conversely, the larger the piece of DNA that can be cloned into the vector within the limits of the essential DNA, the more useful the vector will be for mapping the bacterial genome and creating gene banks. Bacteriophage λ is widely used as a cloning vector. Approximately 40% of the genome is non-essential for bacteriophage propagation. The bacteriophage L1 is approximately the same size as the bacteriophage λ and may have a similar amount of non-essential DNA which can be substituted with cloned DNA. The Tohama I bacteriophage genome was reported by Paloheimo *et al.* (1987) to only be 30 kb in size. In this case, the bacteriophage L1 may be a more suitable bacteriophage from which to create a cloning vector.

The bacteriophage L1, if modified to prevent its spontaneous lysis of the bacterial cells, could be used to introduce new pieces of DNA into the bacterial hosts. If the bacteriophage L1 is a lysogenic bacteriophage with a very sensitive triggering mechanism to enter into the lytic cycle, modifications to this region could create a more stable lysogen. However, if the bacteriophage L1 is a pseudolysogen, it will only reproduce by the lytic pathway. It will be more difficult to construct a lysogenic bacteriophage from a lytic bacteriophage. Lysogenic bacteriophage require the ability to participate in the cross-over events that would integrate the bacteriophage genome into the bacterial chromosome in a location in the bacterial chromosome that would not create a lethal mutation for the bacterial host. In addition, the bacteriophage would have

to have a repressor protein available to prevent the bacteriophage viral, lytic genes from being expressed until necessary or remove the genes responsible for the lytic cycle. The possibility of interchanging DNA within the *Bordetella* species could further illucidate the interreationships of this closely related genus. The possibility of constructing *Bordetella* species with the desired phenotypic traits could be useful in the study of virulence within the genus.

An interesting application of bacteriophage molecular biology has been forwarded for the exploration of the host bacterial chromosomes.

> "The phage genome must code for a protein responsible for DNA packaging into mature phage particles. In the case of *V. cholerae* phage CP-T1, the *pac* gene codes for a 12,900 dalton DNA binding protein, which by analogy with the studies of the *Salmonella* bacteriophage P22, could be an enzyme that recognizes and cleaves at *pac* . A transposon containing the *pac* gene has been constructed, and attempts are being made to use it to obtain high-frequency generalized transducing lysates of *V. cholerae*. The rationale is that if the packaging system has a high affinity for *pac*, then the introduction of this signal into the chromosome would result, upon CP-T1 infection, in producing phage particles with chromosomal DNA, and the frequency at which the markers were present would be dependent upon the distance from the *pac* site and on the orientation of the *pac* site." (Guidolin and Manning 1987)

Plasmid vectors exist for *B. pertussis* (Weiss 1982). A modified plasmid vector incorporating the packaging genes of the bacteriophage L1 could possibly be a means of shuttling DNA from *B. bronchiseptica* to *B. pertussis* or *B. parapertussis*. A limitation of using bacteriophage L1 is its apparent inability to infect *B. pertussis*

strains. However, the bacteriophage Tohama I may be a possible candidate for development of a cloning vector for the *B. pertussis* strains.

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# APPENDIX:

## Media

### Bordet-Gengou Agar (BGA):

The BGA consists of the following :

| | |
|---|---|
| glycerol (glycerine) | 10 ml |
| Milli-Q™ water | 1.0 l |
| Bordet-Gengou agar base (Difco) | 30 g |
| defibrinated sheep or horse blood | 150 ml |

The glycerol was dissolved in the Milli-Q™ water before the addition of the Bordet-Gengou base. After each addition, the media was well mixed. The mixture was autoclaved for 25 minutes. The autoclaved mixture was allowed to sit at room temperature for 15 minutes before being placed into a 52°C water bath for at least 1 hour. The blood, which had been equilibrated to room temperature, was added sterilely to the cooled agar medium. The agar was poured into sterile plates in ~15 ml amounts. The plates were allowed to dry at room temperature overnight and transferred to large metal boxes for storage (see section 2.2).

### Cyclodextrin Solid Medium (CSM) : (Aoyama *et al.* 1986)

The cyclodextrin solid medium consists of two mixtures, the CSM basic medium which is autoclaved and the CSM supplement which is filter sterilized and added to the cooled agar.

CSM basic medium

| | |
|---|---|
| sodium glutamate (mono) | 10.7 g |
| L-proline | 0.24 g |
| NaCl | 2.5 g |
| $KH_2PO_4$ | 0.5 g |
| KCl | 0.2 g |

| | |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 0.1 g |
| $CaCl_2$ | 0.02 g |
| Tris base | 6.1 g |
| casamino acids (Difco) | 0.5 g |
| heptakis (2,6-O-dimethyl)ß-cyclodextrin (Aldrich) | 1.0 g |
| Bacto agar (Difco) | 18.0 g |

The above listed compounds were dissolved in ~ 0.8 l of Milli-Q™ water, the pH of the medium adjusted to pH 7.4 and the volume adjusted to 1 l. The mixture was autoclaved for 15 minutes. The autoclaved media was placed in a 56°C water bath for at least 1 hour before the following CSM supplement was added:

CSM Supplement:

| | |
|---|---|
| L-cysteine (monohydrochloride) | 40 mg |
| $FeSO_4 \cdot 7H_2O$ | 10 mg |
| Ascorbic acid | 20 mg |
| Niacin | 4 mg |
| Reduced glutathione | 150 mg |

The components of the CSM supplement were dissolved in 10 ml of milli-Q™ water, filtered sterilized with a 0.22 µm Nalgene filter and added to the cooled 1 L volume of CSM basic medium. The media was mixed by aggitation and poured in 15-20 ml volumes into 15 x 100 mm sterile disposable Petri dishes, allowed to set at room temperature for 24 hours before storage at 4°C (see section 2.2).

**Stainer-Scholte Broth ($SSB_{50}$)** (Stainer and Scholte 1971)

The Stainer-Scholte broth consists of two separately prepared liquid components, solution A and Solution B, that are mixed after sterilization procedures.

Solution A: (autoclavable)

| | |
|---|---|
| sodium glutamate | 10.7 g |
| Tris (Trizma Base, Sigma) | 6.12 g |
| NaCl | 2.5 g |
| L-Proline | 0.24 g |
| $KH_2PO_4$ (anhyd) | 0.5 g |
| KCl | 0.2 g |
| $MgCl_2 \cdot 6H_2O$ | 0.1 g |
| $CaCl_2 \cdot 2H_2O$ | 0.027 g |

The above listed components were dissolved in ~ 0.9 L of Milli-Q™ water, the pH of the solution adjusted to pH 7.5 with concentrated HCl (about 4 ml HCl per liter solution A) and the volume adjusted to 1 liter. Solution A was autoclaved for 20 minutes at 121°C and 18 psi and allowed to cool to room temperature. The following supplement (solution B) was added to solution A when it had reached room temperature.

Solution B:

| | |
|---|---|
| L-cysteine·HCl | 27 mg |
| $FeSO_4 \cdot 7H_2O$ | 10 mg |
| Ascorbic acid | 20 mg |
| Nicotinic acid | 4 mg |
| Glutathione (reduced ) | 100 mg |

The components of solution B were disolved in 10 ml of Milli-Q™ water filter, sterilized using a 0.22 µm Nalgene filter and aseptically added to 1 liter of solution

A at room temperature. The complete $SSB_{50}$ medium was mixed by swirling the liquid, then dispensed into sterile 500 ml Wheaton bottles and stored at room temperature until use. The medium was used within 1 month of preparation.

**Stainer-Scholte Agar (SSA)** (Peppler 1982)

The Stainer-Scholte agar is base on the Stainer-Scholte broth listed in the preceding section, but with the following modifications :

Modified Solution A: To solidify the medium, 10 g of Nobel agar (Difco) was added to each liter of solution A, autoclaved to sterilize, and cooled in a 56°C waterbath for 1 hour prior to adding the modified solution B.

Modified Solution B: In addition to the supplements in solution B, 1 g of BSA was added to solution B. Solution B with the 1% BSA was filter sterilized using a 0.22 µm Nalgene filter and aseptically added to the modified solution B. The resulting agar, $SSA_{50}$ with a 0.1% BSA supplement, was poured in 15-20 ml portions into 15 x 100 mm sterile, disposable Petri dishes, allowed to set at room temperature for 24 hours before storage at 4°C in large metal tins.